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THE SALTING OUT OF GELATIN INTO TWO LIQUID LAYERS WITH SODIUM CHLORIDE AND OTHER SALTS.

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(From the Chemical Laboratories of Stanford University, Stanford University, California.)

(Accepted for publication, March 29, 1928.)

INTRODUCTION.

Hofmeister (1) and his pupils were the first to conduct extensive studies on the precipitation of proteins by salts. Since that time, the precipitation of proteins or, more particularly in this case, of gelatin has been the object of much study (2). However the fact that under certain conditions proteins may be salted out into two liquid layers seems to have been noted only by Pauli and Rona (3), Spiro (4), and Hardy (2). Pauli and Rona, while studying the effect of salts on the setting and melting points of gelatin solutions, found that if a gelatin solution was precipitated at 30°C. by the addition of a neutral salt and allowed to stand some hours at the same temperature, two liquid layers are formed, the upper containing but little gelatin, the lower being rich in gelatin and correspondingly more viscous. Spiro observed that both casein and gelatin precipitated by sodium sulfate formed liquid layers if allowed to stand in a warm thermostat. Hardy found that by increasing the temperature from 20° to 30°C. it was possible to have two liquid layers in equilibrium with each other in the system edestin, salt, and water. Analysis showed each of the layers contained all three constituents. Spiro further found that the analysis of the lower liquid layer closely agreed with that of the freshly precipitated protein. He points out the similarity between this phenomenon and the salting out of alcohol. In neither case is the phenomenon one of precipitation since "owing to the appropriation of water by the salt" separation into two liquid phases occurs. Each phase contains all the constituents and any alteration in the concentration of any one of the three constituents leads to a readjustment of

the composition and relative amounts of the two phases. Since with alcohol the effect of the electrolyte is not attributable to a specific action of the constituent ions, Spiro points out that such influence of the latter in the salting out of proteins must be regarded as subsidiary. This conception would explain the divergent results obtained in the salting out of proteins with neutral salts when the conditions are not maintained constant.

One source of confusion in the literature dealing with the precipitation of proteins is that there are really two distinct types of precipitation and many writers attempt to correlate data which depend on basically different phenomena. The one type is rightly termed coagulation or precipitation for it resembles the precipitation of suspensoid-hydrosols in that the colloid particles carry an electric charge due to the partial dissociation of their stabilising agent. With this type the precipitating agent is always decomposed or reacts with the stabilising agent. For it to occur, the proteins must be partially ionized, and precipitation is usually brought about by electrolytes of which only small amounts are necessary. The other type is best designated as salting out, for Spiro showed that it is analogous to the salting out of alcohol. Here the agent is not decomposed, the protein may be ionized or not and really large amounts of electrolyte are necessary.

Hardy (2) and Scaffidi (2) applied the phase rule to the systems serum globulin, salts, water; Galeotti (2) has applied it to the systems egg albumin, copper sulfate, water; serum albumin, copper sulfate, water; serum albumin, silver nitrate, water; egg albumin, sodium sulfate, water. Since each may consist of two immiscible components, protein and salt, which are partially miscible in a third component, water, they have been compared with the system succinic nitrile, sodium chloride, water studied by Schreinemakers (5).

Robertson (6) points out that Galeotti finds that solid $CuSO_4 \cdot 5H_2O$, solid protein, and water can coexist, the water being saturated with $CuSO_4 \cdot 5H_2O$ and also containing dissolved protein, as is shown by the fact that dilution of the fluid phase causes further precipitation of protein. From this he argued that protein in solution was not to be regarded as a separate phase for otherwise he would have to postulate four coexistent phases. With regard to this point McBain and Burnett (7) have formulated the statement that for purposes of the

phase rule, a solution of a reversible colloid, no matter how complicated in itself, behaves towards external equilibria as a single phase.

Proteins are not the only colloids which can be salted out, for other substances in the colloidal state exhibit the same phenomena. In fact it is upon this that commercial soap manufacture depends and these phenomena have been far more extensively studied in the case of soaps than for any other class of substance. The results here detailed for gelatin bear a close resemblance to the behaviour of the corresponding solutions of soap as established by the comprehensive work of McBain and his collaborators (7).

EXPERIMENTAL.

Materials.

The salts used were for the most part Kahlbaum's best. Most of the work was done with Eastman ash-free gelatin, though best photographic gelatin (obtained from Dr. Slater Price, Director of the British Photographic Research Association), Coignet's silver label gelatin, and Coignet's gold label gelatin were also used. Analysis of the gelatins for water and ash showed:

Gelatin	Water	Ash
	per cent	*per cent*
Photographic	18.8	0.88
Coignet's gold label	15.4	1.36
Eastman ash-free (used mostly)	11.3	0.08
" " " (sample used in last few experiments)	11.2	0.12

The conductivity of Eastman ash-free gelatin was approximately 10^{-4} mhos in 10 per cent solution indicated that the gelatin contained but very little electrolyte. The effect of any such salt present was negligible for the purpose of this investigation inasmuch as such large quantities of salt are afterwards added.

Hydrogen ion determinations were made with an electrode designed according to Considine (8). Considerable difficulty was met with in determining hydrogen ion, especially in 20 and 30 per cent solutions of gelatin, but results apparently accurate to 0.1 pH could be obtained by replatinizing the electrode after each determination.

Solutions of gelatin containing a given percentage of dry gelatin per 100 gm. of solution were made up as follows. The required amount of gelatin, cut in small pieces, was placed in a beaker with a few crystals of thymol (to prevent mould), covered with the requisite amount of water, and allowed to stand at least 3 hours. The swollen gelatin was placed in an oven and allowed to stand at 35°C. for at least 12 hours. To 100 gm. portions of the resulting solution in 150 cc. glass stoppered bottles varying amounts of salts in the solid form were added. The solution was frequently shaken and kept in the oven at 35°C. till the salt dissolved. Then the desired amount of acid was added and the solution allowed to settle out overnight into two layers. The following day the layers were thoroughly mixed and separation again allowed to take place. This is found necessary for, especially in critical regions, a labile lower layer may be set up on first addition of acid which later, on stirring well, redissolves. A similar effect has been noted by Höber (2). That is, less salt is required at first for production of two liquid layers. This change with time is characteristic of other properties of gelatin, especially osmotic pressure and viscosity. It is possible that the same influences, possibly a change in the amount of aggregation (9), play the same rôle here. Moeller (2) thought the change in amount of precipitated gelatin was due to hydrolysis which would naturally be greater the more the acidity. At any rate to obtain reproducible results, the effect of time must be allowed for. Another property which changes with time is the acidity which has been found to decrease gradually on standing. This alone might well account for decreased separability with time since with all the salts employed an increase in hydrogen ion results in an increase in the gelatin salted out.

After settling has again occurred the layers were separated and analyzed. The upper layer was partially decanted and complete removal facilitated by cooling the system down to 0°C. in an ice bath, whereupon the lower layer gels and the upper layer may be drained off. It is found that this cooling down does not appreciably affect the equilibrium already established, for owing to the high viscosity of the lower layer changes take place very slowly. (In fact a stable lower layer at 60°C. which will dissolve on shaking at 50°C. may be preserved several days without apparent alteration at

room temperature. This is a familiar phenomenon in viscous solutions of soap.) The lower layer was then weighed and both layers analyzed for hydrogen ion for loss at 110°, for loss on gentle ignition, and for residue. Since the amount of acid added is known, and the amount of free acid is known from the pH value, the amount of sorbed or combined acid may be calculated. The free acid is given off at 110° and the combined acid on ignition. Hence the loss at 110° may be corrected to give gelatin content. The residue is sodium chloride.

Qualitative experiments showed that the phenomenon of separation into two liquid layers was a perfectly reversible one which obeyed the phase rule in that a change in temperature, or any of the four components, gelatin, water, salt, or hydrogen ion, resulted in a change in composition and amount of the two layers. The lower layer is always darker in color (unbleached gelatin was used) and more viscous than the upper layer. The lower layer also contained less salt and water and more gelatin than did the upper. Depending upon the conditions the lower layer varied from a clear solution to the dense, opaque, plastic layer which developed when the gelatin in the lower layer was about 30 per cent. Complete separation of upper layer from the viscous lower layer is ordinarily difficult.

On warming up a clear lower layer under the microscope, the first change observed is a clouding of the field with the immediate appearance of many fine indefinite discontinuities. These increase slightly in size and then begin to branch out and intertwine with each other in an indefinite network. (A slight tendency toward spiral formation is noted.) This network increases in fineness and complexity so that the system seems almost granular. Then on cooling down, the first change is the vague formation of a "dried mud flat" appearance which later becomes more pronounced. The cracks are clear while the spaces which they surround exhibit the above mentioned network. On further cooling the cracks widen and the system is composed of droplets in which the network is gradually diminishing. The drops decrease slightly in size and suddenly disappear at a definite temperature. On heating and cooling again the same changes are observed. If before the droplets entirely vanish, the system is heated again, the drops do not change in size but the same changes as noted above go on both inside and outside the drops until finally their outlines become

TABLE I.

Amounts of Salt, or of Salt and the Acid with Common Ion, Added to 100 Gm. of Gelatin Solution, Showing Whether or Not Separation into Two Liquid Layers Occurs, and If So the Ratio of Volume of Upper to Lower Layer.

Gelatin	Salt	Homogeneous		Two liquid layers		
		Salt	Acid	Salt	Acid	Ratio
per cent		*gm.*		*gm.*		
9.0 silver label	KCl	20.0		27.0		
10.0 gold label	$(NH_4)_2SO_4$	10.0		10.0	7 drops*	
10.0 " "	"	10.0		18.0		0.87
10.0 " "	K_2SO_4	10.0	0.2 cc.*	No separation		
10.0 " "	Na_2SO_4	9.0		10.0		1.2
10.0 " "	"	9.0		9.5		0.6
10.0 " "	"	9.0		12.0		1.7
10.0 " "	"	9.0		13.0		2.0
17.6 photographic	"	9.0	0.2 "	10.0	0.4 cc.	
18.1 ash-free	"	9.6	0.6 "	9.6	1.0 "	
10.0 gold label	KNO_3	30.0	11 drops*	35.0	11 drops*	4.1
10.0 " "	"	30.0	11 " *	35.0	24 " *	4.0
10.0 " "	$NaNO_3$	30.0	5 " *	30.0	11 " *	3.0
10.0 " "	"	30.0	5 " *	30.0	24 " *	3.5
10.0 " "	NaCl	26.0		27.0		2.8
10.0 " "	"	10.0		10.0	6.0 " *	
10.0 " "	"	31.0		31.0	2.0 cc.	3.3
10.0 " "	"	19.0	5.0 cc.	20.0	5.0 "	5.5
10.0 " "	"	20.0	4.0 "	20.0	7.5 "	3.1
10.0 " "	"	15.0	6.5 "	15.0	7.0 "	20.0
10.0 " "	"	10.5	10.0 "	15.0	10.0 "	5.0
10.0 " "	"	11.0	7.5 "	11.0	8.5 "	5.0
10.0 " "	"	12.0	20.0 "	12.0	10.0 "	3.9
1.0 " "	"	27.0		20.0	1.0 "	53.0
20.0 " "	"	20.0	7.5 "	20.0	15.0 "	0.52

* Drops of concentrated acid, in all other cases a 1.0 N solution of acid was used.

indistinguishable. At the concentrations studied there is never any formation of any anisotropic phase and both layers are always isotropic.*

* An observation that could not be repeated was that when 50 cc. of a 10 per cent ash-free gelatin solution, to which 10 cc. of N HCl was added, was allowed to evaporate slowly at 35° to about 30 cc., distinct crystals separated out.

Sodium chloride, potassium chloride, sodium sulfate, ammonium sulfate, sodium nitrate, and potassium nitrate were all capable of salting out each of the gelatins employed, but the amounts and acidity required for this varied according to the characteristics of the gelatin. A high ash content in the gelatin seemed to be required for separation with sodium chloride, because on adding this salt, without acid, to 1.1 per cent solution of gelatin free from ash, no separation could be obtained at 35°, even when the solution was saturated with sodium chloride (24 per cent). However, the solution was quite cloudy and on increase of temperature separation did occur. The gelatins containing ash probably contain sulfates which assist the separation. Salting out with potassium sulfate was found impossible, probably owing to its limited solubility.

Table I gives the composition of various mixtures showing which remained homogeneous, and which separated into two liquid layers. It is apparent that the order of strength of anions is $SO_4 > Cl > NO_3$, which is in accord with the Hofmeister or lyophilic series $SO_4 > Cl > C_2H_3O_2 > NO_3 > Br > I > SCN$.

Many workers (Moeller, Fenn, Loeb and Loeb, Michaelis and Davidsohn, Pauli, and Posternak (2), Lewith, Bancroft, Bogue, Morner, Mines, Procter (10)) have noted that an acid solution aids the salting out of many proteins. However only a few (Chick and Martin, Hardy, Mellanby, and Sörensen and Höyrup (2)) have recognized that the hydrogen ion also constitutes a component, thus making a quaternary system composed of hydrogen ion, water, sodium chloride, and gelatin. The salts as well as the acid added change the pH (11), and since the relations between salt, acid, and gelatin are probably complex, the only satisfactory method of determining pH would be electrometrically. Unfortunately with most of the experiments carried out the pH was not closely enough determined to give an accurate picture of the distribution of the hydrogen ion between the upper and lower layers, as they do not differ markedly in pH. It was noted however that usually the more completely the gelatin was separated the greater was the hydrogen ion concentration of the upper layer as compared with the lower one. Also the presence of much salt seemed to diminish the hydrogen ion concentration of the upper layer relative to that of the lower. It apparently is possible to have either a system

TABLE II.

Analyses of Systems.

Total HCl in system	Composition of system*		Composition of upper layer*				Composition of lower layer*			
	NaCl	Gelatin	NaCl	Gelatin	pH	Weight of layer	NaCl	Gelatin	pH	Weight of layer
gm.	*per cent*	*per cent*	*per cent*	*per cent*		*gm.*	*per cent*	*per cent*		*gm.*
0.000	23.4	11.0	No separation. The solution is saturated with NaCl				On warming separation occurs			
0.051	20.8	7.5	Separation about to occur							
0.077	18.5	8.1	No separation							
0.077	19.9	9.9	Separation about to occur							
0.077†	20.0	8.0	19.9	6.7	4.1	116.3			4.1	8.7
0.077†	20.1	7.9	20.5	6.9		113.6	17.7	17.4		12.4
0.077			20.5	7.2	4.1	117.6			4.0	8.5
0.077	20.7	9.0	20.8	8.6		124.0	17.6	19.2		5.0
0.077	21.5	9.2	21.7	8.0	4.1	112.2	19.2	18.8	3.8	13.3
0.077			22.7	4.8	4.2	100.4			4.2	27.5
0.077			23.2	4.3	4.3	99.4			4.3	30.7
0.077	23.3	7.2	24.4	3.2		102.5	19.4	20.6		30.0
0.077	23.3	7.3	24.5	3.4	4.0	102.7	19.4	20.8	3.8	30.1
0.11	20.6	7.5	21.5	4.0	3.2	102.8	17.3	20.5		26.9
0.145	15.8	8.2	No separation							
0.145	16.3	7.9	No separation							
0.18	15.8	8.2	16.2	6.1	3.0	100.2	13.5	18.9		19.8
0.18	15.8	8.2	15.9	7.5	3.5	118.5	13.7	19.5	3.5	7.3
0.18	16.7	8.3	17.3	5.2	3.5	101.1	13.0	24.5	3.3	18.4
0.18	18.5	8.1	19.8	2.7		93.7	14.7	24.5		30.3
0.18	20.5	6.9	21.9	1.2	3.3	102.4	14.9	28.5	3.3	26.8
0.18			21.9	0.4	3.25	106.6				24.8
0.18	21.3	7.2	23.6	0.77		103.7	16.0	27.4		31.8
0.22†	13.1	8.7	13.2	7.9	3.3	106.2	11.0	19.4	3.1	7.9
	16.3	7.9	17.2	4.0	2.9	95.8	13.2	22.1		26.4
0.23	13.1	7.2	13.3	3.5	2.9	92.5	10.1	21.7	2.9	22.3
0.23	13.2	8.3	Separation about to occur							
0.27	15.8	8.0	17.25	1.5	3.2	94.6	11.3	27.5	3.15	31.1
0.27	16.1	8.2	17.5	1.6	3.0	94.2	11.4	28.9	2.9	29.7
0.27	20.2	7.4	22.1	0.64	3.2	102.0	13.6	30.9	3.1	29.5
0.29	10.6	7.9	Separation about to occur							
0.29	10.7	8.9	11.1	6.1	2.9	86.3	9.4	17.2	3.0	26.0
0.31	9.9	9.0	10.1	7.7	2.5	92.7	8.9	15.5	2.7	18.6
0.35	13.2	8.3	14.0	4.6	2.0	94.5	9.7	26.4	2.0	18.9

* Water constitutes the remainder of the system and may be calculated if desired by subtraction.

† If the system had been stirred, the analyzed layers would have mixed homogeneously.

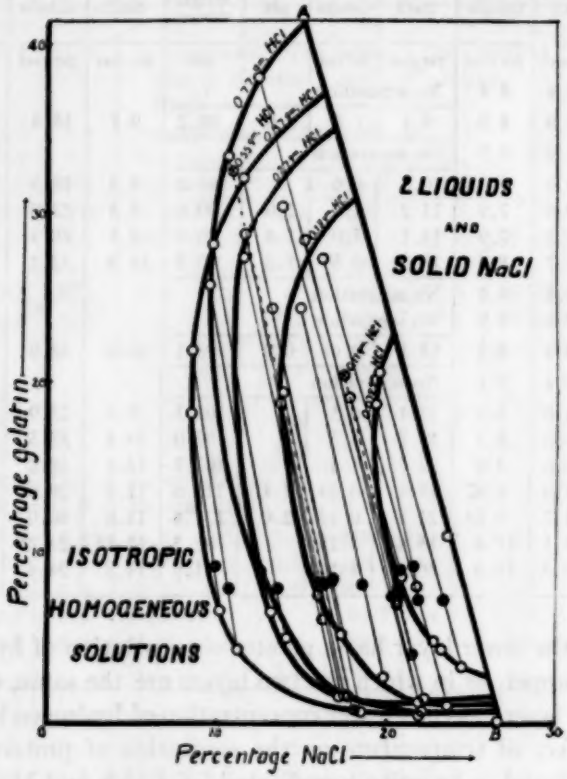
TABLE II—*Concluded.*

Total HCl in system	Composition of system*		Composition of upper layer*				Composition of lower layer*			
	NaCl	Gelatin	NaCl	Gelatin	pH	Weight of layer	NaCl	Gelatin	pH	Weight of layer
gm.	*per cent*	*per cent*	*per cent*	*per cent*		*gm.*	*per cent*	*per cent*		*gm.*
0.37	8.4	8.4	No separation							
0.37†	8.9	8.9	9.1	7.3	2.0	88.2	9.7	15.4	2.0	24.4
0.37	9.4	8.9	No separation							
0.37	9.8	9.2	10.1	6.6		86.8	8.3	18.3		25.0
0.37	10.6	7.9	11.2	4.3	2.0	90.6	8.3	22.0		23.0
0.37	13.5	7.9	14.1	5.0	1.4	96.9	9.5	28.3	1.35	13.8
0.37	16.7	8.4	18.5	0.59	1.3	90.5	11.3	32.2	1.6	29.7
0.55	8.4	8.4	No separation							
0.55	9.4	8.9	No separation							
0.55	16.4	8.2	18.1	0.68	0.9	93.1	10.9	32.6	1.1	28.7
0.77	10.4	8.6	No separation							
0.77	12.5	8.9	13.1	6.5		103.5	9.3	25.9		15.2
0.77	16.2	8.1	17.7	1.5		97.0	10.4	33.5		26.8
0.77	20.6	8.0	22.25	1.4		107.7	12.2	38.2		23.5
0.037	17.0	0.87	17.1	0.29	2.1	115.6	11.5	28.9		2.4
0.037	21.7	0.85	21.8	0.12	2.0	122.4	11.6	40.0		2.3
0.55	13.1	17.4	15.4	5.25		38.5	11.55	23.25		78.8
0.55	16.3	16.3	20.1	1.16		41.9	14.3	24.4		80.4

in which the lower layer has a greater concentration of hydrogen ion than the upper, or in which the two layers are the same, or in which the upper layer has the greater concentration of hydrogen ion.

The effect of temperature on the separation of proteins by salts has been found to be quite complicated (*cf.* Chick and Martin, Fenn, Galeotti, Guerrini, Scaffidi, Hardy, Hofmeister, and Spiro (2)) and Lewith (10). As Fenn points out, there is much confusion and the data in the literature are conflicting, some writers finding for a given protein a positive temperature coefficient, others a negative one, and still others a negligible one. This is what would be expected from an inspection of our phase rule diagram depending on what fraction was chosen for comparison. Actually in many cases there is a reversal of the temperature coefficient at a definite temperature which varies according to the amount of salt and hydrogen ion in the system.

When conditions are such that there is considerable gelatin in the upper layer (5 per cent), an increase in temperature markedly assists in salting out. When there is but a small amount of gelatin in the upper layer (1 per cent) both raising and lowering of temperature may



○ Composition of one of the liquid phases.
● Total composition of the system.

FIG. 1. Isotherms at 35° showing the salting out of aqeuous gelatin by sodium chloride into two liquid layers, one isotherm for each concentration of hydrochloric acid. The region to the left represents insufficient salt for any separation; the middle region two liquid layers; and the region to the right excess of salt in solid form.

aid salting out. A further increase in temperature may even cause the solution of that separated at a moderate temperature. Undoubtedly the isotherms for various temperatures are not straight and

parallel with each other but exhibit maxima and minima and cross each other in a way like those found for globulin, water, and magnesium sulfate by Galeotti (2).

Table II gives the analysis of different systems studied using ash-free gelatin, sodium chloride, and hydrochloric acid, and Fig. 1 shows

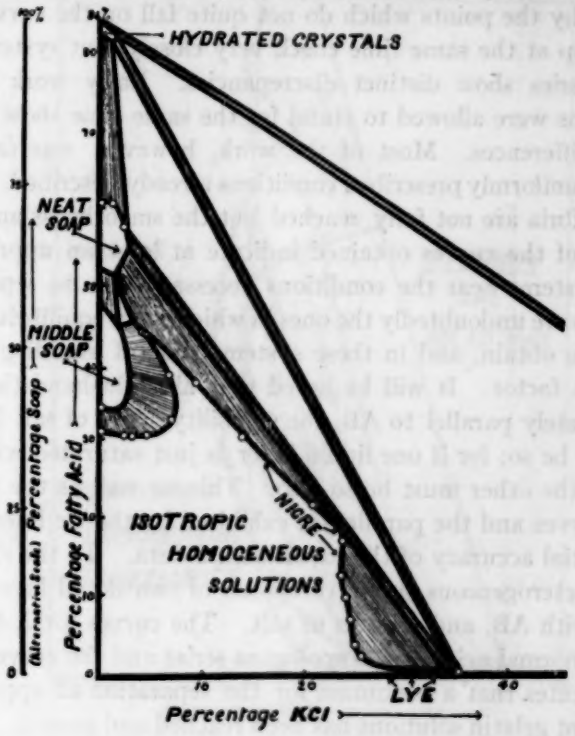


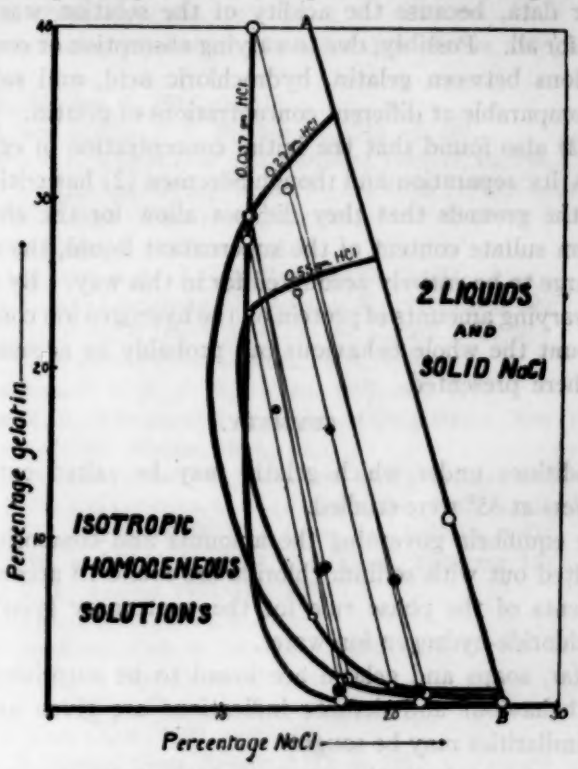
FIG. 2. Phase rule diagram at 90°, potassium laurate-potassium chloride-water; concentrations are expressed in grams of fatty anhydride and grams of potassium chloride per 100 gm. of total system. The two liquid systems, nigre-lye, correspond to the areas in Figs. 1 and 3.

the isotherms at 35°C. of the systems containing the same amounts of hydrochloric acid. The clear region on the left where little salt is present, represents a single isotropic phase. As the amount of salt is increased separation into two isotropic liquid phases occurs which would have nearly the same composition if the initial gelatin solution used was about 11 per cent. As more salt is added, the composition

and amount of the two layers varies until the solutions become saturated with salt. The solubility of sodium chloride in gelatin was determined only for an 11 per cent gelatin solution and it is assumed that the line AB, extended, to which all the isotherms are extrapolated, is a straight line. The experimental limits of error are indicated by the points which do not quite fall on the curves. Systems set up at the same time check very closely, but systems whose history varies show distinct discrepancies. Early work in which the systems were allowed to stand for the same time show the most marked differences. Most of the work, however, was carried out under the uniformly prescribed conditions already described. Possibly true equilibria are not fully reached but the smoothness and relative positions of the curves obtained indicate at least an approximation to it. Systems near the conditions necessary just to separate into two layers are undoubtedly the ones in which exact equilibrium is most difficult to obtain, and in these systems time of standing is a very important factor. It will be noted that all right-hand tie lines are approximately parallel to AB, the solubility curve of salt in gelatine and must be so; for if one liquid layer is just saturated with sodium chloride, the other must be so also. This, as well as the continuity of the curves and the parallelism exhibited by the tie lines, confirms the essential accuracy of the experimental data. To the right of AB lies the heterogeneous sytem composed of two liquid layers in equilibrium with AB, and crystals of salt. The curves for 2, 5, 7.5, and 10 cc. of normal acid form homologous series and the curve for 20 cc. acid indicates that a maximum for the separation of approximately 10 per cent gelatin solutions has been reached and passed. If sodium chloride were more soluble it is evident that no acid would be necessary for separation. Hydrochloric acid alone will not bring about the salting out, nor can the ash-free gelatin be salted out with sodium chloride in a solution rendered alkaline with sodium hydroxide.

The phase rule diagram for potassium laurate-potassium chloride-water at 90° found by McBain and Field (7) is shown in Fig. 2. The shape and location of the bay bounding the region wherein the two isotropic liquid phases, nigre and lye, coexist, strikingly resemble the bay found for gelatin-sodium chloride-water. It is of no great consequence that gelatin itself is not a definite chemical entity because

mixtures of soaps likewise yield phase rule diagrams which closely resemble those of single soaps (McBain (12)). Obviously since gelatin is so very like soap, as indicated by our diagrams, if anisotropic regions corresponding to neat soap and middle soap are possible



○ Composition of one of the liquid phases.
● Total composition of the system.

FIG. 3. Isotherms at 35° showing the salting out of aqueous gelatin by sodium chloride into two liquid layers, one isotherm for each concentration of hydrochloric acid. The total content of gelatin in the system is widely varied in these experiments.

for gelatin, they must be sought in regions where the concentration of gelatin is higher than 40 per cent.

Fig. 3 shows the differences obtained using widely different initial concentrations of gelatin. At first sight these curves might appear

to belie the applicability of the phase rule, since for any one upper or lower layer, the composition of the other ought to be fixed according to the phase rule regardless of the relative amounts of the two layers in equilibrium. However, this point cannot be tested fully by these particular data, because the acidity of the solution was not kept the same for all. Possibly, due to varying absorption or combination, the relations between gelatin, hydrochloric acid, and salt are not strictly comparable at different concentrations of gelatin. Chick and Martin (2) also found that the initial concentration of egg albumin influences its separation and though Sörensen (2) has criticized their data on the grounds that they did not allow for the alteration in ammonium sulfate content of the supernatant liquid, the differences are too large to be entirely accounted for in this way. By taking the effect of varying amounts of protein on the hydrogen ion concentration into account the whole behaviour can probably be accounted for in the way here presented.

SUMMARY.

1. Conditions under which gelatin may be salted out into two liquid layers at 35° were studied.

2. The equilibria governing the amounts and composition of the lavers salted out with sodium chloride are found to accord with the requirements of the phase rule for the quaternary system gelatin-sodium chloride-hydrogen ion-water.

3. So far, soaps and gelatin are found to be surprisingly similar in their behaviour and definite indications are given as to where further similarities may be sought.

4. It is evident from this work that the term "coagulation" as ordinarily applied to the salting out of proteins is definitely a misnomer.

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HEMOLYSIS OF CHICKEN BLOOD.

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This paper is concerned with the kinetics of hemolysis of chicken red cells by the simple hemolysins, saponin, sodium taurocholate, and sodium oleate. The action of these lysins has been extensively studied on mammalian red cells, but quantitative investigations on the nucleated cell are wanting.

Methods.

The methods used in this work are almost identical with those described by Ponder (1, 2). They will be briefly sketched here.

The standard blood cell suspension is usually taken as a 2.5 per cent suspension of erythrocytes, twice washed in 0.85 per cent NaCl. In this work, however, a double strength suspension, 5 per cent of chicken blood cells, was found to give a more distinct end-point and was therefore used throughout. The suspension is always made up from freshly drawn oxalated blood, and used within a few hours after preparation. The estimation of the time taken for the lysis is carried out in a constant temperature water bath, made with glass sides; this permits the contents of the tubes to be seen by transmitted light without the removal of the tubes from their environment.

The white lined background of the bath permits accurate determinations of the end-point for non-nucleated cells. But with the nucleated chicken erythrocyte the sharp end-point as found in the hemolysis of human cells is not obtainable, the lack of sharpness being due to suspended nuclei and ghosts remaining after the hemolysis is complete. The ruled background thus proves of little value. This necessitates the establishment of a new end-point for complete hemolysis of the nucleated red cell. This end-point is naturally more arbitrary than the definite one found in the hemolysis of non-nucleated cells, and it is only with much practice that consistent values are obtained in our experiments. To aid in these determinations two tubes for comparison are placed in the bath; one tube contains a suspension of cells in the same dilution but without the lysin, the other tube contains an

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example of complete hemolysis of the nucleated blood. The temperature of the bath is kept at the constant temperature of 25°C.

A series of stock dilutions of saponin are required. These dilutions are labelled 1 in 10,000, 1 in 20,000 up to 1 in 70,000. Any intermediate dilutions can be made as required. To keep the amount of the hemolytic agent which comes in contact with the cells in round numbers, the following method is used. A known weight of saponin is dissolved in sufficient 0.85 per cent NaCl to make it 2.5 times the strength indicated. For example, the dilution labelled 1 in 10,000 is really 1 in 4,000 saponin. When 0.8 cc. of this solution is taken and to it is added 0.8 cc. of saline and finally 0.4 cc. of the blood suspension, the resulting dilution is 1 in 10,000 with a final volume of 2 cc. These saponin stock dilutions keep without loss of hemolytic power for about a week in well stoppered bottles and in a cool place.

The dilutions of taurocholate are prepared as follows. The sodium taurocholate after having been weighed in a stoppered weighing bottle is dissolved in a quantity of 0.8 per cent NaCl to make a 2.5 per cent solution. From this solution the other necessary dilutions are made as required. As Ponder points out, among other precautions necessary to observe when working with taurocholate, the solution must be used as soon as the dilutions are made, because upon standing even a short time the taurocholate undergoes a change in physical state and loses some of its hemolytic power.

The same method as outlined for taurocholate preparation is used in the making of the sodium oleate solution. The dilutions are made from the 2.5 per cent solution of oleate.

In this hemolytic work great care must be exercised that all glassware is clean. The cleansing is done in distilled water; the glass is then exposed to live steam and dried in a hot air oven.

To illustrate how a determination is made an example follows. Of the saponin solution marked 1 in 10,000, 0.8 cc. is placed in a small dry test-tube of clear white glass (1.5 cm. by 10 cm.). To this is added 0.8 cc. of 0.85 per cent NaCl solution; the tube is then placed in the water bath. In the same bath, there are placed a vessel containing the blood suspension to be added to the lysin, and a tube containing a graduated pipette immersed in 0.85 per cent NaCl solution. When the suspension and the dilution of the lysin have acquired the temperature of the bath, 0.4 cc. of the blood suspension is added with the warmed pipette. The time between the addition of the blood suspension and the completion of the hemolysis is noted with a stop-watch.

This procedure is carried out for the different dilutions of the lysin. A time-dilution curve (as shown in Fig. 1) is thus obtained by plotting the dilution of the lysin against the time taken by it for the completion of lysis of the quantity of suspension used.

The observed curve is then fitted with the expression,

$$t = \frac{1}{\kappa} \log \frac{c}{c - x} \qquad (1)$$

in which t is the time in minutes required for the completion of the lysis, c the initial concentration of lysin in the system measured in milligrams, x the amount in milligrams of lysin used up in producing the lysis, and κ a constant.

To determine the value x, we find the asymptote where $t = \infty$ and $c = x$. This is found first by inspection and then by trial; the value of c corresponding to the asymptote gives the value of x, the constant κ is then found from the above expression. All these curves have been determined several times, but only a representative one for each lysin is given here.

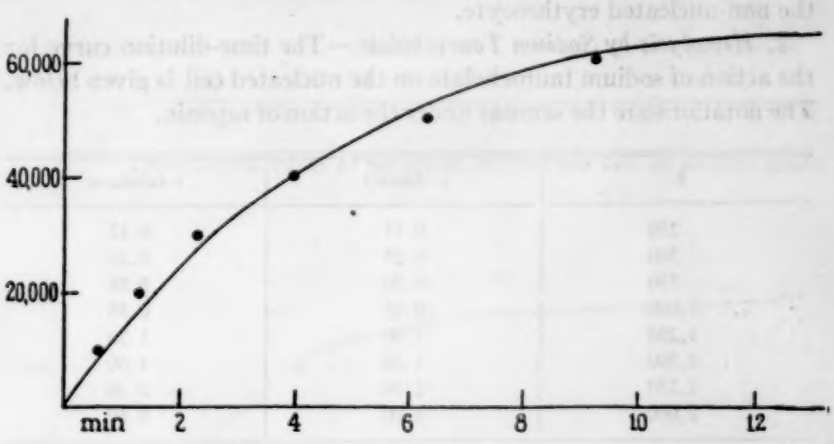

FIG. 1. Time-dilution curve of nucleated chicken red cell by saponin.

RESULTS.

1. Hemolysis by Saponin.—The relations between t, the time for complete hemolysis of the chicken cell suspension, and δ, the dilution of the saponin acting on the cells in the system of 25°C., is expressed below.

δ	t- observek	t- calculated
10,000	0.6	0.7
20,000	1.3	1.6
30,000	2.3	2.6
40,000	4.0	4.0
50,000	6.3	5.8
60,000	9.3	8.8
70,000	23.5	29.0

The values of t are calculated from the values $t = \infty$ and $\delta = 72{,}000$, hence $x = 0.277$ and $\kappa = 0.203$. This calculated curve is given in Fig. 1, and the observed points plotted as small circles. From this graph it is obvious that the observed curve is described by the expression (1) with a goodly degree of accuracy; it may therefore be concluded that the time-dilution curve for the action of saponin on the nucleated red cell is very similar to the curve given by saponin on the non-nucleated erythrocyte.

2. Hemolysis by Sodium Taurocholate.—The time-dilution curve for the action of sodium taurocholate on the nucleated cell is given below. The notations are the same as under the action of saponin.

δ	t- observed	t- calculated
250	0.15	0.17
500	0.25	0.36
750	0.50	0.58
1,000	0.75	0.85
1,250	1.00	1.20
1,500	1.58	1.60
1,750	2.50	2.30
2,000	6.00	6.60

The values of t are calculated from $x = 0.9091$ and $\kappa = 0.715$, where $t = \infty$ and δ is 2,200. It is found (Fig. 2) that the formula fits the observed curve. The curve is similar in almost every respect to that with the saponin, except in the range of effective dilutions. Whereas 1 in 72,000 of saponin is the dilution for hemolysis in infinite time, 1 in 2,200 of the taurocholate is necessary.

3. Hemolysis by Sodium Oleate.—In the action of sodium oleate on the nucleated chicken red cell a new point, not met in the case of the other lysins, must be considered. In high concentrations this lysin first attacks the corpuscular membrane, thus causing hemolysis as we have been considering it. The lysin then continues its action and destroys the nuclei and ghosts, and as a result a clear end-point, as in non-nucleated cell hemolysis, is reached. Thus from the one experiment two end-points for each dilution are found within a limited range of activity. From these two sets of data, two curves can be obtained, with a different value of κ and of $t = \infty$ for each. One curve is the

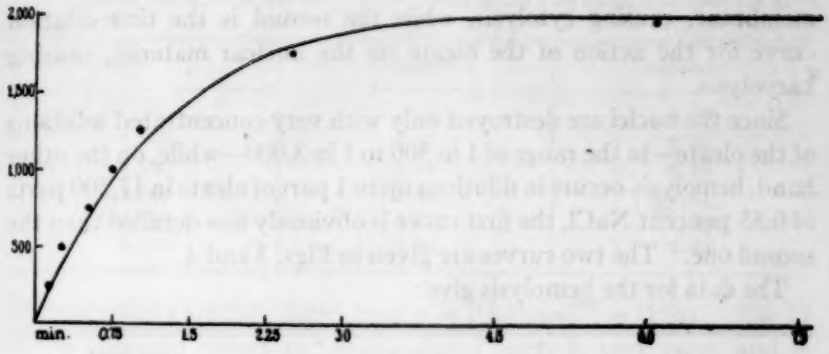

FIG. 2. Time-dilution curve of nucleated chicken red cell by sodium taurocholate.

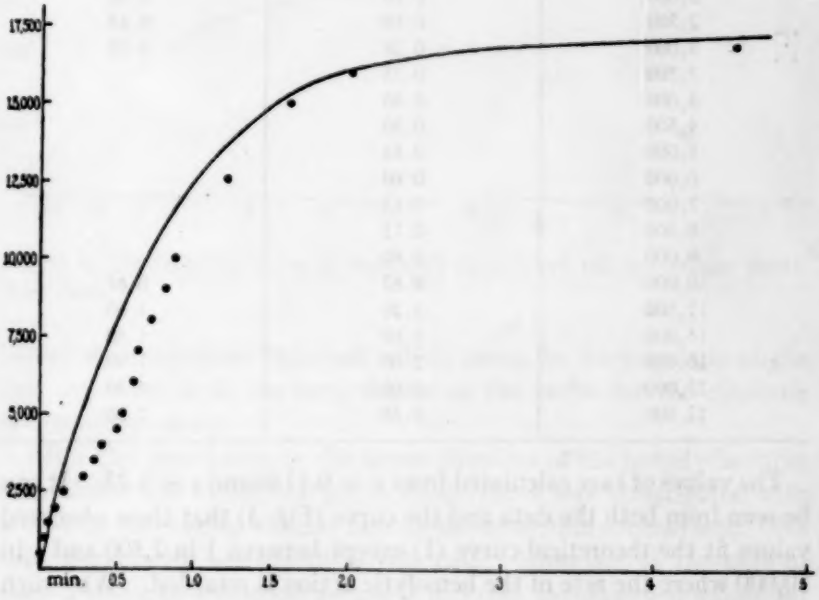

FIG. 3. Time-dilution curve of nucleated chicken red cell by sodium oleate. Hemolysis or cytolysis.

time-dilution curve for the action of the lysin on the corpuscular membrane, causing cytolysis, while the second is the time-dilution curve for the action of the oleate on the nuclear material, causing karyolysis.

Since the nuclei are destroyed only with very concentrated solutions of the oleate—in the range of 1 in 500 to 1 in 3,000—while, on the other hand, hemolysis occurs in dilutions up to 1 part of oleate in 17,500 parts of 0.85 per cent NaCl, the first curve is obviously less detailed than the second one. The two curves are given in Figs. 3 and 4.

The data for the hemolysis give:

δ	t- observed	t- calculated
500	0.02	0.02
1,000	0.03	0.05
1,500	0.07	0.07
2,000	0.10	0.10
2,500	0.16	0.13
3,000	0.28	0.15
3,500	0.35	
4,000	0.40	
4,500	0.50	
5,000	0.53	
6,000	0.60	
7,000	0.63	
8,000	0.71	
9,000	0.80	
10,000	0.87	0.67
12,500	1.20	1.00
15,000	1.60	1.50
16,000	2.00	1.90
17,000	5.00	4.50
17,500	8.50	7.80

The values of t are calculated from $x = 0.1136$ and $\kappa = 1.23$. It can be seen from both the data and the curve (Fig. 3) that these observed values fit the theoretical curve (1) except between 1 in 2,500 and 1 in 10,000 where the rate of the hemolytic action is retarded. Why such an irregularity should occur cannot be definitely stated at this time. However, a somewhat similar case is found in the hemolysis of the non-nucleated red cell when sodium glycocholate is used (3).

The data for the curve (Fig. 4) formed from the clear end-points are:

δ	t- observed	t-calculated
500	0.10	0.29
1,000	0.67	0.69
1,500	1.16	1.16
2,000	2.00	1.82
2,500	3.25	2.81
3,000	9.30	9.40

The values of t are calculated from $x = 0.625$ and $\kappa = 0.541$. The fit of the observed values to the calculated curve is very good, and in the light of the difficulty in judging the end-point the curve is really

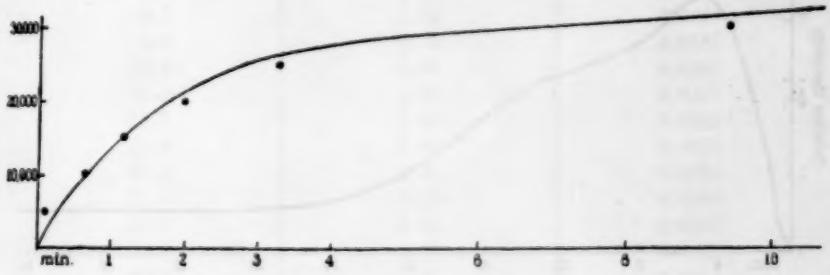

FIG. 4. Time-dilution curve of nucleated chicken red cell by sodium oleate. Karyolysis.

better than might be expected. This curve for karyolysis is of the first order and is of the same degree as the curve for the cytolytic action of the oleate.

After this peculiarity in the lower dilutions of the hemolysis curve with oleate was found, the taurocholate curve was investigated with a view to discovering whether any similar deflection occurs there. No such irregularity was found in this case. However, in the case of taurocholate, in a dilution around 1 in 2,000, the observed values for t do not always agree with the calculated one, but from time to time vary, with the result that this dilution gives a cluster of points rather than a constant one. This interesting phenomenon is under study at present.

Method of Determining Viscosity.

The determinations of viscosity are made by the Ostwald method. The system is placed in the constant temperature bath at 25°C. and with this method consecutive readings are made rapidly and without removing the fluid under observation from the bath.

The lysin, saline, and blood suspension are in the same concentration as used in the test-tube for determining the time-dilution curves of the lysin; *i.e.*, 2 cc. of the selected dilution of the lysin is put into the apparatus, then 2 cc. of 0.85 per cent NaCl is added. After these have reached the temperature of the bath, 1 cc. of the blood suspension is added and immediately the system sucked up into the calibrated bulb. The time consumed for the fluid to leave the bulb is recorded by a

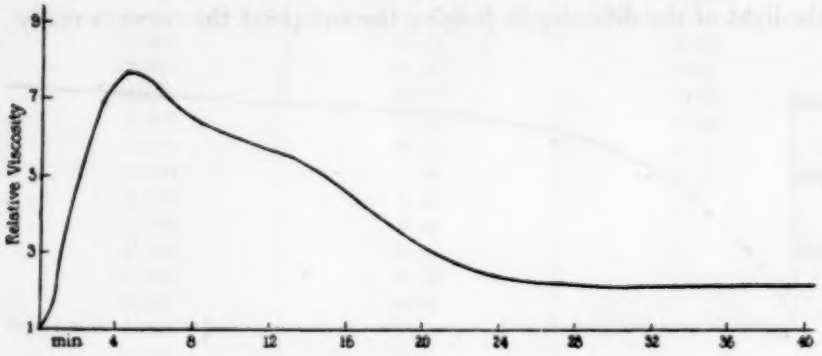

FIG. 5. Time-viscosity curve of sodium taurocholate-cell system.

stop-watch. These readings are continued until the viscosity becomes constant. The system is gently blown through prior to each reading to insure thorough mixing. The viscosity of water is taken as unity and the relative viscosities are found by comparison with that of water; the absolute viscosity at 25°C. is found by multiplying the relative viscosity by the constant 0.009. The time-viscosity curves are plotted with the relative viscosity on the ordinate and the moment of time at which the fluid is released from the top of the bulb on the abscissa.

For the sake of completeness, viscosity changes were looked for in connection with the hemolysis of the nucleated red cell by saponin, but none were found. The viscosity changes noted in connection with the hemolysis by taurocholate and oleate, respectively, occur only after the hemolysis of the cells is mostly, if not entirely, completed.

1. Viscosity in Sodium Taurocholate Blood System.—Viscosity changes with taurocholate are found with dilutions ranging between 1 in 100 and 1 in 600. It was found most convenient with our apparatus to take a medium strength solution, hence 1 in 400 is the strength of the sodium taurocholate used.

Although the curve is plotted from readings taken as frequently as possible, only part of the data is given.

Time	Relative viscosity	Absolute viscosity at 25°C.
min.		
0.3	1.21	0.0109
0.9	2.14	0.0193
1.7	4.86	0.0438
3.0	6.50	0.0575
4.6	7.36	0.0662
6.5	7.00	0.0630
8.0	6.50	0.0585
10.0	6.00	0.0540
13.0	5.50	0.0495
16.0	4.50	0.0405
19.5	3.00	0.0270
23.4	2.57	0.0231
25.9	2.29	0.0206
35.0	2.14	0.0193
60.0	2.14	0.0193
90.0	2.07	0.0186
120.0	2.07	0.0186

As can be seen from the figure (Fig. 5) the viscosity very rapidly reaches a maximum (7 or so times that of water) within 5 or 6 minutes after the blood suspension and the lysin have been mixed. The viscosity then drops, rather rapidly at first, then more slowly, until a point where the system is practically twice as viscous as water and there it remains.

2. Viscosity with Sodium Oleate.—The same type curve is found in respect to the sodium oleate and chicken cell suspension as is given by the action of sodium taurocholate. However, since the oleate has a stronger hemolytic activity than the taurocholate, a weaker concentration is used. A dilution of 1 in 1,200 is found to be about the best for this work.

Time	Relative viscosity	Absolute viscosity at 25°C.
min.		
0.3	1.21	0.0109
0.9	2.14	0.0193
1.6	5.00	0.0450
3.0	9.64	0.0868
5.5	9.57	0.0861
8.0	8.64	0.0778
10.3	7.64	0.0688
14.3	7.14	0.0643
16.3	6.21	0.0559
17.9	5.64	0.0508
19.3	5.07	0.0456
20.9	4.36	0.0392
23.4	3.71	0.0334
26.5	3.21	0.0289
29.3	2.86	0.0253
32.6	2.57	0.0231
35.9	2.50	0.0225
70.0	2.50	0.0225

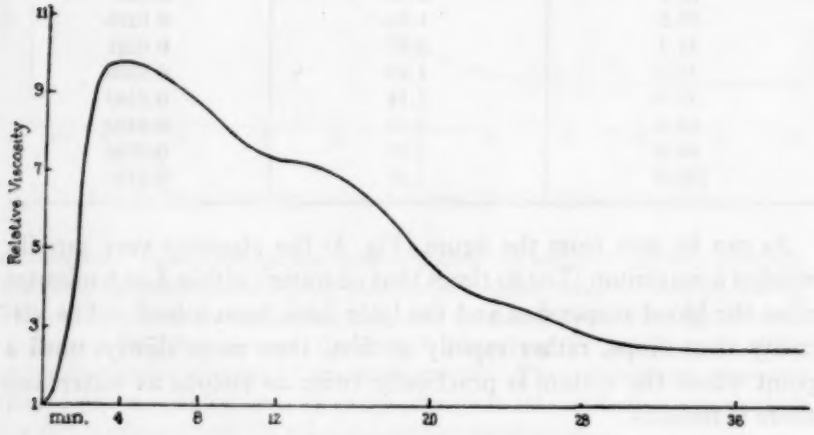

FIG. 6. Time-viscosity curve of sodium oleate-cell system.

In hemolysis by high concentrations of oleate, the nuclei are rapidly destroyed (karyolysis) before the viscosity change appears and the red fluid is transparent. However, within 10 minutes after hemolysis the fluid becomes cloudy once more.

As can be seen from the curve (Fig. 6) the system becomes almost 10 times as viscous as water after about 5 minutes. Then, as in the case of taurocholate, the curve gradually descends and in this case reaches a minimum viscosity after about 35 minutes. By a comparison of the oleate and the taurocholate curves, although the oleate solution is one-third the strength of the taurocholate solution, it will be seen that they are of the same general form and doubtlessly represent the same type of phenomenon.

The viscosity changes, both in case of oleate and taurocholate, do not occur until after the hemolysis of the nucleated chicken red cell is complete. The fact that taurocholate and oleate form loose compounds with the hemoglobin of the cells may have some relation to these viscosity changes.

Microscopic Action of Lysins.

Although no actual measurements of the changes in the shape, size, etc., of the nucleated erythrocytes were made during the action of the lysins upon them, the cells were frequently examined microscopically.

The chicken erythrocyte in 0.85 per cent NaCl appears ellipsoidal in flat view, except that each end of the cell is distinctly pointed. The nucleus appears to be a biconvex body in the center of the cell. After the lysin is added to the cell, it gradually loses its pointed ends and its ellipsoidal shape and becomes circular in flat view. After assuming this spherical shape, it gradually fades from view, leaving what appears to be a smaller nucleus in the center of a spherical ghost-cell. These observations hold for all three lysins used, and point to the fact that the mechanism of the hemolysis of the nucleated blood cell is similar to that found in the hemolysis of the non-nucleated mammalian erythrocyte (4).

SUMMARY AND CONCLUSIONS.

1. The time-dilution curves are given for the hemolytic action of saponin, sodium taurocholate, and sodium oleate on nucleated chicken erythrocytes.

2. Saponin and sodium taurocholate cause hemolysis but leave the nuclei and ghosts in suspension, thereby making the end-point of

hemolysis more arbitrary than the clear end-point for non-nucleated cell hemolysis.

3. The curves of hemolysis by saponin and taurocholate are shown to be of the same nature as are found in the hemolysis of non-nucleated cells.

4. Sodium oleate causes first hemolysis and then, in the stronger solutions, causes karyolysis. Two pairs of values for κ and $c = \infty$ are thus obtainable for the same reaction, one pair for the destruction of corpuscular membrane, the other pair for the destruction of the nucleus.

5. Viscosity changes are found in the lysin-cell system with strong concentrations of sodium taurocholate and sodium oleate. Time-viscosity curves are given for these changes.

6. Microscopically, the action of these lysins on the nucleated chicken red cell appears to be similar to their action on the non-nucleated erythrocytes.

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ELECTRIC IMPEDANCE OF SUSPENSIONS OF SPHERES.

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I.

The electric resistance of a suspension of homogeneous spheres is given by the relation

$$\frac{r_1/r - 1}{r_1/r + 2} = \rho \frac{r_1/r_2 - 1}{r_1/r_2 + 2} \quad (1)$$

in which r, r_1, and r_2 are the resistances[2] of the suspension, the suspending, and suspended phases respectively, and ρ is the volume concentration of the suspended phase.

Since emulsions, suspensions of living cells, and colloidal particles have an interfacial surface layer which is markedly different from both the interior and exterior phases, the suspended phase can only be considered homogeneous under a few special conditions. As has been shown by Maxwell (9), §313, a sphere of radius a_2 and resistance r_2 surrounded by a concentric spherical shell of internal radius a_2,

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[1] This equation has been variously derived in the early theory of several physical problems but it often disagrees with the data (Lowry (8)) for atomic and molecular phenomena—probably because the assumptions underlying it are not valid in these cases. It has, however, been found by Fricke and Morse (3) to apply accurately to the resistance of cream up to 62 per cent volume concentration of butter fat, and its use is probably justified for the resistance of suspensions of spheres which are of much larger than atomic dimensions. A simple derivation is given by Maxwell (9), §314.

[2] Unless otherwise stated, all resistances, reactances, and impedances are specific, *i.e.*, for a centimeter cube.

external radius a_3, and resistance r_3, Fig. 1, can be replaced by a homogeneous sphere of radius a_3 and equivalent resistance $\hat{r}_2$ where

$$\hat{r}_2 = r_3 \frac{(2r_2 + r_3)\, a_3^3 + (r_2 - r_3)\, a_2^3}{(2r_2 + r_3)\, a_3^3 - 2(r_2 - r_3)\, a_2^3}.$$

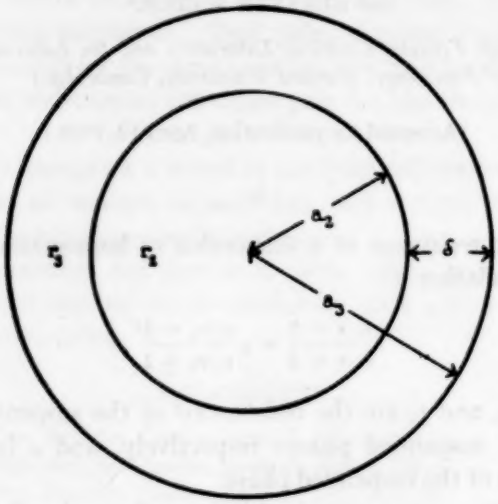

FIG. 1.

When $a_2 = a_3 - \delta = a - \delta$, where δ is a small quantity such that higher powers than the first can be neglected,

$$\hat{r}_2 = \frac{r_2 + \frac{\delta\, r_3}{a}\left(1 - \frac{r_2}{r_3}\right)}{1 - \frac{2\,\delta}{a}\left(1 - \frac{r_2}{r_3}\right)}.$$

If further, r_2/r_3 is small compared with unity,

$$\hat{r}_2 = r_2 + \frac{\delta\, r_3}{a}. \tag{2}$$

Assuming that the current flow in the surface layer is radial, then δr_3, which is a resistance per unit area, may be replaced by z_2, a complex

impedance per unit area given in alternating current vector notation (1, 7, 11) by

$$z_3 = r_3 + jx_3 \tag{3}$$

where the resistance per unit area r_3 is in series with a reactance per unit area x_3 and j is the imaginary operator. Substituting z_3 of Eq. (3) for δr_3 in Eq. (2) and replacing $\hat{r}_2$ by $\hat{z}_2$ the equivalent complex impedance of the sphere, we have

$$\hat{z}_2 = r_2 + z_3/a = r_2 + r_3/a + jx_3/a. \tag{4}$$

Solving Eq. (1) for r,

$$r = r_1 \frac{(1-\rho)\, r_1 + (2+\rho)\, r_2}{(1+2\rho)\, r_1 + 2(1-\rho)\, r_2}.$$

Substituting $\hat{z}_2$ for r_2 and replacing r by z, the complex impedance of the suspension,

$$z = r_1 \frac{(1-\rho)\, r_1 + (2+\rho)\, (r_2 + r_3/a) + (2+\rho)\, jx_3/a}{(1+2\rho)\, r_1 + 2(1-\rho)\, (r_2 + r_3/a) + 2(1-\rho)\, jx_3/a}. \tag{5}$$

On the assumption that r_1 and r_2 are constant, when $z_3 \to \infty$, z is a pure resistance

$$r_0 = r_1 \frac{2+\rho}{2(1-\rho)}; \tag{6}$$

and when $z_3 \to 0$, z is again a pure resistance

$$r_\infty = r_1 \frac{(1-\rho)\, r_1 + (2+\rho)\, r_2}{(1+2\rho)\, r_1 + 2(1-\rho)\, r_2}. \tag{7}$$

These are two cases in which the spheres may be considered homogeneous.

Solving Eq. (5) for z_3

$$z_3 = \frac{z - r_\infty}{r_0 - z}\, \gamma \tag{8}$$

where

$$\gamma = r_2 a \left[1 + \frac{(1+2\rho)}{2(1-\rho)} \cdot \frac{r_1}{r_2}\right]. \tag{9}$$

When $r_2 = 3r_1$, and ρ varies from 0.2 to 0.4, the second term in the brackets varies from 0.3 to 0.5. Thus γ is of the same order of magnitude as $r_2 a$ and more or less constant for many cases.

Separating z into its resistance and reactance components,

$$z = r + jx,$$

the absolute value or magnitude of $|z_3|$ is given by

$$|z_3|^2 = \frac{(r - r_\infty)^2 + x^2}{(r_0 - r)^2 + x^2} \gamma^2. \tag{10}$$

In the special case where r_3 is constant or zero, it can be shown that

$$(r - r_\infty)(r_0 - r) = x^2.$$

Then

$$\frac{x_3^2}{\gamma^2} = \frac{r - r_\infty}{r_0 - r}; \tag{11}$$

and also

$$\frac{x_3^2}{\gamma^2} = \frac{|z|^2 - r_\infty^2}{r_0^2 - |z|^2}. \tag{12}$$

II.

When r_2 is finite and z_3 varies with the frequency n of the measuring current so that when $n \to 0$, $z_3 \to \infty$, and when $n \to \infty$, $z_3 \to 0$, then $|z|$ from Eq. (5) as a function of n is shown in Fig. 2.

If r_3 varies such that

$$r_3 = m x_3 \tag{13}$$

then it can be shown that as x_3 varies from 0 to ∞ the locus of the end of the impedance vector when plotted on the complex plane is an arc of a circle as shown in Fig. 3. Also the ratio of the chords a and b is

$$\frac{a}{b} = \frac{|z_3|}{\gamma}. \tag{14}$$

At the points r_∞ and r_0, the slopes of the tangents are respectively $1/m$ and $-1/m$.

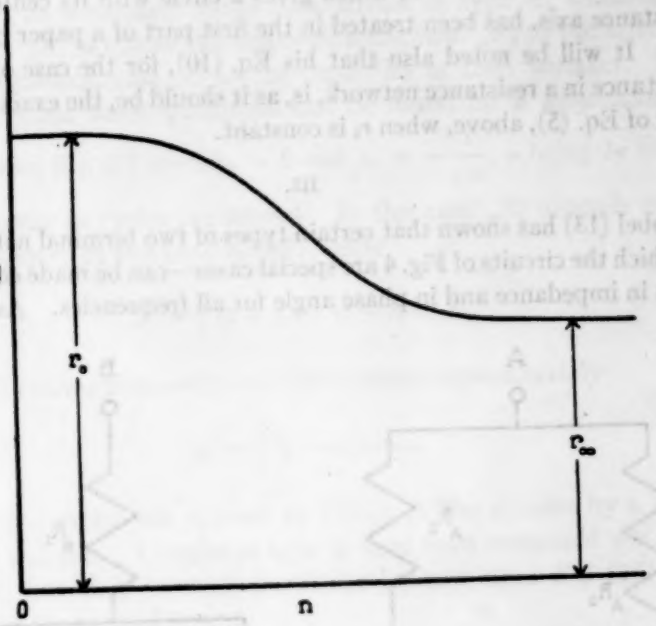
|z|
r_o
r_∞
0
n

FIG. 2.

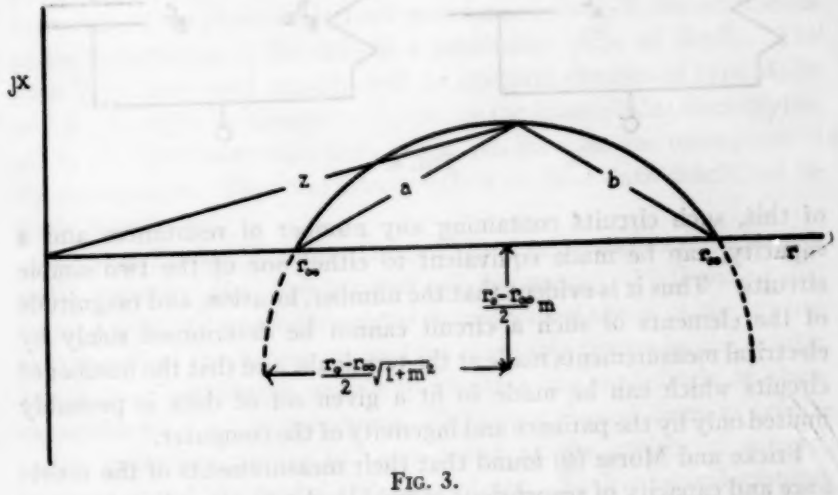
jx
z
a
b
r_∞
r_∞
r
$\frac{r_o - r_\infty}{2} m$
$\frac{r_o - r_\infty}{2}\sqrt{1+m^2}$

FIG. 3.

The case where $m = 0$, which gives a circle with its center on the resistance axis, has been treated in the first part of a paper by Carter (2). It will be noted also that his Eq. (10), for the case of a single reactance in a resistance network, is, as it should be, the exact counterpart of Eq. (5), above, when r_3 is constant.

III.

Zobel (13) has shown that certain types of two terminal networks—of which the circuits of Fig. 4 are special cases—can be made equivalent both in impedance and in phase angle for all frequencies. As a result

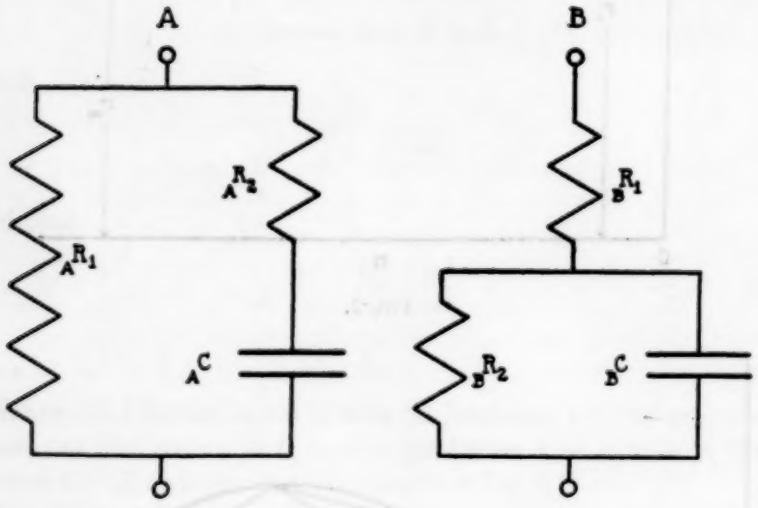

FIG. 4.

of this, such circuits containing any number of resistances and a capacity, can be made equivalent to either one of the two simple circuits. Thus it is evident that the number, location, and magnitude of the elements of such a circuit cannot be determined solely by electrical measurements made at the terminals, and that the number of circuits which can be made to fit a given set of data is probably limited only by the patience and ingenuity of the computer.

Fricke and Morse (6) found that their measurements of the resistance and capacity of suspensions of red blood cells at various frequen-

cies could be accurately fitted to a circuit of type A, where they thought of ${}_AR_1$ as due to the suspending medium, ${}_AR_2$ to the interiors of the corpuscles, and ${}_AC$ to the capacities at their surfaces. The values of these three quantities can be found for a suspension of spheres from Eq. (5) when $r_3 = 0$ and $x_3 = -\frac{1}{c_3\omega}$, ω being 2π times the frequency in cycles per second. In this case ${}_AR_1$ depends on r_1, and ρ, ${}_AR_2$ on r_1, as well as on r_2 and ρ, while

$$ {}_AC = \frac{1}{2}(2 + r_1/r_2)(1 - r_1/r_2)\, ac_2, $$

For small volume concentrations this becomes approximately

$$ {}_AC = \frac{3}{2}(1 - r_1/r_2)\, ac_2, \tag{15} $$

which is the expression derived by Fricke (4) for spheres by a quite different analysis. Circuits of type B have been computed which fit the data, but they have no particular interest since Eq. (15) was derived on the basis of type A.

In his work on the impedance of tissues Philippson (10) assumed a circuit of type B where ${}_BR_2$ and ${}_BC$ represented the resistances and capacities of the physiological cell membranes, and ${}_BR_1$ the resistances of the protoplasm of the cells in a centimeter cube of tissue. The data have been used equally well to compute circuits of type A, in which ${}_AR_1$ might be thought to be due to the intercellular electrolytes, ${}_AC$ to the membrane capacities, while ${}_AR_2$ involves the resistances of the protoplasm. Thus the interpretation of these data should not be made intuitively.

Whereas Fricke (5) found the red blood cell membrane to have a static capacity (*i.e.* independent of frequency), Philippson (10) found a capacity which for animal tissues varied about as the inverse square root of the frequency, and for vegetable tissues as the inverse fourth root. Although unable to measure the magnitude of these capacities for a single membrane, Philippson classed them as polarization capacities similar to those found at the surface of contact between metal electrodes and electrolytes. Such physical systems have a polarization resistance such that the phase angle, ϕ_3, of the

combination is more or less independent of frequency and often in the neighborhood of 45° (see Wolff (12)). Then in Eq. (13), $\phi_3 = ctn^{-1} m$. On the assumption that this angle was 45°, Philippson calculated the series polarization resistance although he took no account of it in the computations of the capacities from the data. In Eq. (5), the polarization resistance would be represented by r_3 as a series resistance per unit area. Since r_3 would be a function of frequency, more or less proportional to x_3, it would be difficult if not impossible to compute it from impedance data alone without making assumptions which are at present unwarranted. If, however, measurements are made of both the resistance and reactance of a suspension—with a capacity bridge, for instance—it becomes possible to determine both r_3 and x_3 from Eq. (5).

SUMMARY.

A general expression has been derived for the electric impedance of a suspension of spheres each having a homogeneous non-reactive interior and a thin surface layer with both resistance and reactance. The applications and limitations of impedance measurements on such suspensions are discussed.

The author very much appreciates the interest and assistance of Mr. K. S. Johnson of the Bell Telephone Laboratories, Inc., and of Professor E. L. Chaffee and Professor W. J. Crozier.

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ELECTRIC IMPEDANCE OF SUSPENSIONS OF ARBACIA EGGS.

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In work done several years ago on the heat production of the *Arbacia* egg at fertilization and during early development (11) the suggestion was made that the phenomena observed might be due to changes in the cortex. Inasmuch as the heat data can give no clue to the action of component parts of the egg, another means of analysis was sought which might give an answer to the various questions raised. The work of Fricke and Morse (3) and Philippson (10) suggested that the impedance of suspensions of eggs to various frequencies of alternating current might yield valuable facts regarding the electric capacity and resistance of the surface of the egg, and the resistance of the interior, and the changes of these three quantities at fertilization and during development.

Apparatus.

Although it was first planned to use a capacity bridge to measure both the resistance and the capacity of the suspensions of *Arbacia* eggs it was found after considerable work that the bridge had several very serious defects for this particular work. In the first place it became very complex as the range of frequencies to be investigated was extended (3). This made it necessary to devote an undue amount of time to the taking of each reading and the changing from one frequency to another. In the second place, unless one were willing to go to the very ultimate in amplification it was necessary to use an undesirably high current density in the electrolytic cell. In this work the aim was to keep the potential difference across each egg so low that the

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current probably would in no way affect it. From these and other considerations it was decided to use a variation of the "ammeter-voltmeter" method of measuring resistances. This method, while less sensitive and giving less data, is on the other hand direct reading and can be made independent of the frequency over a wide range. At first glance it would appear that this was the method used by Philippson (9), but it soon becomes evident that what he used was essentially a comparison method which involved many of the inaccuracies and troubles of the bridge method and gave only the results and accuracy of the direct reading method which has been used in the present work. A small alternating current of the desired frequency was sent through an electrolytic cell containing the suspension and simultaneous measurements were made of the current through the cell and the potential difference across it. The ratio of these two gave the magnitude of the impedance of the cell at that frequency but did not, however, give the phase angle.

Oscillator.—The source of supply of alternating current was a vacuum tube oscillator. This oscillator is of the type known as "tuned plate circuit" and while the type is well known (2) and very commonly used, this particular instrument has several unusual features of design and operation. Although its range can probably be extended in both directions, it has been used entirely between the frequencies of one thousand and fifteen million cycles per second. It is completely shielded electrostatically and is controlled only by switches on the panel over the entire range of frequencies. It has a readily changed but constant output and its frequency calibration has remained remarkably constant. While it is usual to take the output from such an oscillator by means of a coil loosely coupled to the tuned circuit, it was not found advantageous to do so in this oscillator. In addition to the necessity of switching both the grid and plate coils as the frequency was changed this output coil would have to be changed and it would also be a decided advantage in such a case to be able to vary the coupling. These needs would have made the oscillator so much slower and less flexible that it was decided to take the output from the main oscillatory circuit as is shown in Fig. 1. The inductance L' is very small as compared with the main oscillator inductance L but carries the oscillatory current flowing through the condenser.

Over the frequency range of any single inductance L the potential difference across L' remains comparatively constant. This output is connected to the electrolytic cell through a resistance sufficiently high to prevent reactions of the output back on the oscillatory circuit.

Frequency.—A two-turn coil was loosely coupled to the coil L'

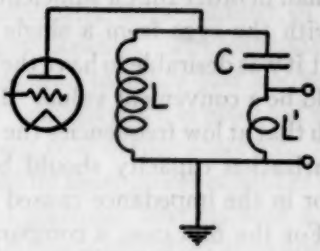

FIG. 1.

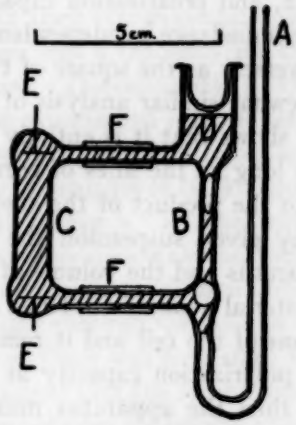

FIG. 2.

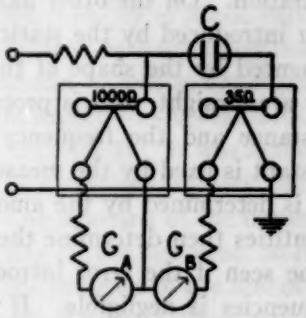

FIG. 3.

FIG. 1. Method of taking output from vacuum tube oscillator.

FIG. 2. Electrolytic cell and stirrer.

FIG. 3. Circuit for measurement of impedance.

within the oscillator. When the frequency was to be measured, connections from this coil were made through the shielding to a similar coil which was coupled to a General Radio Precision Wavemeter. The resonance point of the wavemeter was determined by means of a vacuum tube voltmeter connected directly across the variable condenser.

Electrolytic Cell.—The factors governing the design of the cell were several from both the biological and the electrical standpoints. In order to prevent settling of the suspended eggs it was necessary to have continuous but gentle stirring. It was also necessary to provide for adequate oxygenation of the suspension. The total volume of the cell should be small in order that a sufficient volume concentration may be obtained with the eggs from a single specimen. From the electrical standpoint it was desirable to have the cell constant such that the impedance would be a convenient value. It was also necessary to have the design such that at low frequencies the error in the impedance caused by the polarization capacity should be small while at high frequencies the error in the impedance caused by the static capacity should be small. For the first case, a comparatively simple analysis assuming a straight line current flow shows that for any given frequency, specific resistance of electrolyte, and polarization capacity per unit area of electrode, the error in the impedance is independent of the area of the electrodes but varies inversely as the square of their separation. On the other hand, a somewhat similar analysis of the error introduced by the static capacity shows that it is entirely uninfluenced by the shape of the cell—as long as the lines of current flow are straight—but is proportional to the product of the specific resistance and the frequency. For any given suspension the cell constant is fixed by the measuring apparatus and the volume of the cell is determined by the amount of material available. These two quantities then determine the dimensions of the cell and it remains to be seen if the error introduced by polarization capacity at low frequencies is negligible. If it is not then the apparatus must be modified, other material used, or corrections introduced. If the error due to the static capacity is not small enough to be neglected at the high frequencies there is only one alternative, that of lowering the specific resistance of the suspension, which may demand the use of other material in certain cases. From these considerations the cell shown in Fig. 2 was designed and found to fullfil all of the requirements

A supply of saturated air at constant pressure was connected to tube A. The bubbles rising in B forced the suspension in that tube before them and gave rise to a circulation in a counter-clockwise direction, giving an entirely adequate stirring and at the same time allowing

a thorough oxygenation of the suspension. Considerable difficulty was experienced from persistance of bubbles; the surface tension of suspension was considerably different from that of sea water as was shown both by this persistance of bubbles at D and by increased size of the bubbles rising in tube B, Fig. 2. If these bubbles were not broken as soon as they appeared at the surface a froth was formed which contained a sufficiently large number of eggs to change the volume concentration noticeably. The only satisfactory method of breaking these bubbles was to place just above the surface of the suspension, at D, a "U"-shaped piece of glass rod which had previously been rubbed on the oily nose of the observer. The electrolytic cell proper C was connected by short pieces of rubber tubing at F F. The electrodes E E were of platinum covered with a very light and very fine deposit of platinum black plated on at as low a current density as possible. It was rather important that these electrodes be placed with their planes containing the axes of the side tubes, for otherwise there was a tendency for the eggs to pile up either above or below them. The cell used in this work has a constant of 5.85. No difficulty was experienced from either polarization capacity or static capacity, a perfectly constant impedance being obtained with sea water between the frequencies of 1000 cycles per second and 15 million cycles per second, D, Fig. 4. The pressure of the air supply was so regulated that there was always at least one bubble in the tube B. It was found that under these conditions about 10 cc. of air at atmospheric pressure passed through the cell per minute. The lowering of the impedance when there was no bubble in tube B was only 4 per cent, so it was assumed that the error introduced by the presence of the film of solution between the bubble and the glass wall of B was entirely negligible. The volume of suspension required was 4 cc.

Measuring Apparatus.—The measuring apparatus consisted of two Western Electric vacuum thermocouples connected to two Leeds and Northrup type *R* low resistance galvanometers. The deflections of both galvanometers were read by light spots thrown on the same scale.

In the vacuum thermocouples there is a small filament of metal or carbon known as the heater through which the current to be measured passes. Attached to the center of this heater element are two wires of dissimilar materials forming a thermocouple to measure the tem-

perature rise in the heater wire resulting from the heat produced by the current to be measured. This whole arrangement is placed in a glass bulb which is then evacuated. The evacuation greatly increases the sensitivity and at the same time makes the arrangement independent of small changes of room temperature. It is found that for small currents the E.M.F. of the thermo-junction is directly proportional to the square of the current flowing through the heater. For this work a couple having a 1000 ohm heater was used to measure the potential drop across the cell and the other thermocouple. This second thermocouple was used to measure the current through the cell and had a resistance of 34 ohms. It was soon found that this arrangement would not work at high frequencies, due to the fact that both galvanometers had a considerable capacity to ground while neither one was at ground potential. This state of affairs led to a more and more effective short circuiting of one-half of each of the heater elements as the frequency was increased beyond a certain point. The solution was to use only one-half of each heater element, Fig. 3—one of the thermocouple terminals serving for the other heater connection. In this way both galvanometers were at the same potential as the grounded side of the output from the oscillator. The high resistance thermocouple then served as an alternating current voltmeter and the low resistance thermocouple as an alternating current ammeter. When but one-half of a heater element is used the sensitivity of the device is multiplied by a factor of $\sqrt{2}$ when used as a voltmeter and divided by the same factor when used as an ammeter. From the constants of the thermocouples and the galvanometers it was found that

$$|Z| = 150 \sqrt{s_A/s_B} \text{ ohms.}$$

Experimentally it was found that

$$|Z| = 151 \sqrt{s_A/s_B} \text{ ohms,}$$

where s_A and s_B are the deflections of the voltmeter and ammeter galvanometers respectively, and $|Z|$ is the absolute value of the impedance.

The apparatus was checked over the entire range of frequencies by means of a specially designed shielded resistance whose impedance

was constant up to 10 million cycles, being in error by only 1.6 per cent at that frequency. It was found that the impedance of the electrolytic cell containing sea water, curve D, Fig. 4, was constant up to the highest frequency used, about 15 million cycles. The apparatus has also been checked by measuring the impedance of condensers and inductances.

In actual practice, the square root of the ratio of deflections of the two galvanometers was multiplied by a factor of 151 to give the impedance of the electrolytic cell plus the ammeter heater. This latter was a pure resistance of 18.4 ohms. At both the very high and the very low frequencies, the cell filled with a suspension of eggs had a zero phase angle and the resistance of the series heater could be subtracted directly to give the resistance of the cell suspension, but at the intermediate frequencies the phase angle was not zero and this fact had to be taken into account in the determination of the impedance of the cell suspension.

With the apparatus in its final form it was possible to take readings every 40 seconds over long periods and half of this time interval was consumed in recording the data. The actual impedance measurements had an accuracy of about 1 per cent. For full scale deflections the current through the cell was 0.002 ampere, and the potential drop across it 0.3 volt.

Data.

Of the living animal cells which were available, the eggs of the Echinoderm *Arbacia punctulata* seemed the most satisfactory because of their uniformly spherical shape, their hardiness, and the possibility of using large numbers of them and having them keep "in step" for a considerable period after fertilization. The amount of investigation to which these eggs have been subjected and the availability of a moderate volume of eggs from a single specimen added to their desirability.

Over a thousand measurements of the impedances of suspensions of biological material were made at the Marine Biological Laboratory at Woods Hole during the summer of 1927. Although all of the analysis has been confined to the results on *Arbacia* eggs some data were taken on red blood corpuscles and on the eggs of *Asterias* and *Fundulus*.

Procedure.

The filament of the oscillator vacuum tube was usually turned on for at least 15 minutes in order to allow it to reach equilibrium before any readings were taken. Then a run was taken with the standard resistance to check the constancy of calibration of the thermocouples and galvanometers. A second run was taken with the electrolytic cell filled with sea water to determine the conductivity of the sea water and make certain that the error due to polarization capacity of the electrodes was negligible.

The ovaries of the *Arbacia* were removed entire from the specimen and placed in a finger bowl half filled with sea water. After standing for several minutes they were gently shaken with forceps and removed. The suspension was then carefuly poured through wet unbleached muslin and allowed to settle. After settling the clear sea water was either pipetted or poured off, or else the eggs were brought to the center of the finger bowl by moving it in a circle and then pipetted into fresh sea water. In order to get rid of as much jelly as possible the suspension was gently poured from one finger bowl to another several times at each washing. Sometimes the eggs were centrifuged by hand just enough to throw them down to get the concentration desired before removing them to the electrolytic cell, but this procedure was avoided wherever possible. As soon as the suspension was placed in the cell the air line was connected and the stirring and aeration continued without interruption until the end of the experiment.

A set of readings usually consisted of the zero readings of both galvanometers with the oscillator disconnected and then the readings of both galvanometers at seven or eight different frequencies. After half a dozen or so sets of readings, the eggs were fertilized by the thorough stirring in of a small amount of "dry" sperm and readings continued until after first cleavage. In one or two runs the observations were continued up to 3 hours after fertilization but never longer than that. Very small samples were frequently removed from the cell for microscopic examination. At the end of the run the suspension was removed from the cell and centrifuged to determine the volume concentration of the eggs.

An entirely satisfactory technique for the handling of the eggs has

not been worked out as yet and while normal development was obtained in not over half of the runs made it was considered expedient to take some data on eggs—normal or abnormal—rather than to have normal development and no data. Polyspermy was probably the most common cause of abnormality and was not recognized as such

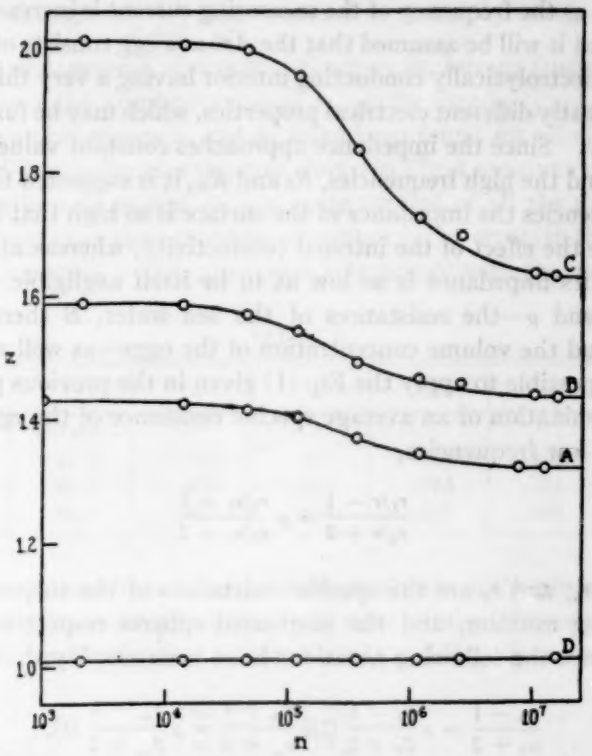

FIG. 4. Impedance (Z, arbitrary units) *vs.* log frequency (n, cycles per second) for sea water and *Arbacia* egg suspensions.

for some time due to the failure of the eggs to raise membranes although fertilized. The reason for this may be that in the concentrated suspensions used a slight cytolysis would elevate the osmotic pressure to such a degree that the membranes would not lift until the suspension was diluted with sea water.

Computations.

The general type of impedance variation with frequency is shown in the three curves of Fig. 4 for suspensions of *Arbacia* eggs in sea water. This change in impedance with frequency can mean but one thing, namely, some element or elements in the egg have an impedance which decreases as the frequency of the measuring current is increased. For the present it will be assumed that the *Arbacia* egg consists of a homogeneous electrolytically conducting interior having a very thin surface layer of vastly different electrical properties, which may be functions of frequency. Since the impedance approaches constant values at both the low and the high frequencies, R_0 and R_∞, it is suggested that at the low frequencies the impedance of the surface is so high that it renders negligible the effect of the internal conductivity, whereas at high frequencies its impedance is so low as to be itself negligible. Having R_w, R_B, and ρ—the resistances of the sea water, B thermocouple heater, and the volume concentration of the eggs—as well as R_0 and R_∞, it is possible to apply the Eq. (1) given in the previous paper[1] for the determination of an average specific resistance of the egg at both high and low frequencies,

$$\frac{r_1/r - 1}{r_1/r + 2} = \rho \frac{r_1/r_2 - 1}{r_1/r_2 + 2} \tag{1}$$

where r, r_1, and r_2 are the specific resistances of the suspension, the suspending medium, and the suspended spheres respectively. For convenience the following notations have been employed,

$$\frac{\alpha_0 - 1}{\alpha_0 + 2} = \rho \frac{\beta_0 - 1}{\beta_0 + 2} \text{ (2)}, \quad \frac{\alpha_\infty - 1}{\alpha_\infty + 2} = \rho \frac{\beta_\infty - 1}{\beta_\infty + 2} \text{ (3)},$$

where

$$\alpha_0 = \left(\frac{r_1}{r}\right)_0 = \frac{R_w - R_B}{R_0 - R_B}, \beta_0 = \left(\frac{r_1}{r_2}\right)_0,$$

$$\alpha_\infty = \left(\frac{r_1}{r}\right)_\infty = \frac{R_w - R_B}{R_\infty - R_w}, \beta_\infty = \left(\frac{r_1}{r_2}\right)_\infty.$$

[1] Cole, K. S., *J. Gen. Physiol.*, 1928, xii, 29.

For rapid calculation a curve of α *vs.* $\frac{\alpha-1}{\alpha+2}$ has been found very useful. From the α given by the data as shown above, the corresponding $\frac{\alpha-1}{\alpha+2}$ is found from this curve. When divided by the observed ρ, this gives $\frac{\beta-1}{\beta+2}$ from which β is found by using the same curve in the reverse direction. Since r_1 is known (usually about 25 ohm cm.) the absolute value of r_2 can be found.

By the above means β_0 and β_∞ have been found for all of the data taken and their values for the runs shown in Fig. 4 are given in Table I. The variations in β_0 and β_∞ are quite typical of all the data taken and are far greater than could be accounted for by errors in the electrical measurements. This conclusion is in accord with the observation

TABLE I.

	ρ	α_0	β_0	α_∞	β_∞	ρ'
	per cent					per cent
A	22.2	.733	.08	.804	.282	19.6
B	38.0	.676	.275	.763	.452	24.2
C	44.3	.477	.028	.647	.308	42.5

that the variations in both β_0 and β_∞ were always in the same direction from one run to another but that both were relatively constant during any one run. Eliminating ρ from Eq. (2) and Eq. (3) we have

$$\frac{\alpha_0-1}{\alpha_0+2}=K\frac{\alpha_\infty-1}{\alpha_\infty+2} \tag{4}$$

where K involves only β_0 and β_∞ and should thus be independent of ρ. A plot of $\frac{\alpha_0-1}{\alpha_0+2}$ against $\frac{\alpha_\infty-1}{\alpha_\infty+2}$ for all of the data gave a straight line passing through the origin with $K = 1.41$. This was the final check which verified the electrical measurements and placed all of the blame for the variations on the measurements of ρ, the volume concentration of eggs.

In Table I, ρ' is the volume concentration calculated from the

electrical data on the assumption of Eq. (6) of the previous paper[1] that $\beta_0 = 0$ or that at low frequencies the eggs are very poor conductors. The more reliable values of ρ indicate that this assumption is very close to the truth. For this case, from Eq. (4) we have

$$K = -\frac{1}{2}\frac{\beta_\infty + 2}{\beta_\infty - 1} \tag{5}$$

from which $\beta_\infty = r_1/r_2 = 0.28$. The specific resistance of the interior of the egg is then about 3.6[2] times that of the sea water or 90 ohm cm. As said before, ρ was obtained by centrifuging the suspension at the end of the run. This was never very satisfactory as it was desired to allow the eggs to develop further in order to determine their condition. Consequently the suspensions were centrifuged only enough to give what was thought to be "constant volume." The results show that this was not the case and several other methods of determining the volume concentration have been suggested and will be tried in future work. The formula has never been accurately checked with *Arbacia* simply because of this difficulty in the determination of ρ.

As a first approximation it has been assumed that the surface of the egg acts like a pure capacity, with $r_3 = 0$—corresponding to Fricke's conclusion for the red blood corpuscle and Philippson's assumptions for tissue. On this hypothesis, take Eq. (12) of the previous paper[1]

$$\frac{x_2}{\gamma} = \sqrt{\frac{|z|^2 - r_\infty^2}{r_0^2 - |z|^2}} = s, \tag{6}$$

and s may now be computed directly from the impedance on suspensions of *Arbacia* eggs, as has been done in Table II and Fig. 5 for the runs plotted in Fig. 4. The straight lines are drawn arbitrarily with an intercept of 1.0 at $n = 3.10^5$ and a slope of -0.5 in each case. It will be noticed that the slope of each of these lines is about -0.5 and that s is not greatly affected by the volume concentration.

$|Z|$ has been computed from the two sets of data of Fricke and Morse

[2] Fricke and Morse (3) find the resistance of the interior of the red blood corpuscle to be 3.5 times that of the plasma.

(3) and the values of s are given in Table III and plotted in Fig. 6. The slope of the line is -0.93.

Philippson's data (10) gives slopes of about -0.5 for animal tissues and -0.75 for vegetable tissues.

TABLE II.

A			B			C		
ν	$\|Z\|$	s	ν	$\|Z\|$	s	ν	$\|Z\|$	s
$1.0 \cdot 10^3$	1.433		$2.0 \cdot 10^3$	1.589		$2.0 \cdot 10^3$	2.015	
$1.35 \cdot 10^4$	1.425	3.84	$1.38 \cdot 10^4$	1.586		$1.38 \cdot 10^4$	2.008	
$4.45 \cdot 10^4$	1.414	2.3	$4.55 \cdot 10^4$	1.571	2.64	$4.55 \cdot 10^4$	1.99	5.1
$1.15 \cdot 10^5$	1.403	1.71	$1.16 \cdot 10^5$	1.542	1.56	$1.16 \cdot 10^5$	1.955	2.83
$3.48 \cdot 10^5$	1.368	.865	$3.59 \cdot 10^5$	1.491	.77	$3.59 \cdot 10^5$	1.834	1.08
$1.15 \cdot 10^6$	1.343	.51	$1.13 \cdot 10^6$	1.465	.51	$1.13 \cdot 10^6$	1.723	.56
$7.33 \cdot 10^6$	1.322		$2.5 \cdot 10^6$	1.454	.41	$2.5 \cdot 10^6$	1.695	.44
$1.2 \cdot 10^7$	1.317		$1.0 \cdot 10^7$	1.436		$1.0 \cdot 10^7$	1.634	
			$1.5 \cdot 10^7$	1.429		$1.5 \cdot 10^7$	1.626	

TABLE III.

ν	$\|Z\|$	s	$\|Z\|$	s
$.087 \cdot 10^6$	191		332	
.833	180	2.03	317	2.04
1.17	172	1.43	306	1.435
1.52	166	1.155	297	1.135
2.04	157	.875	286	.88
3.04	146	.618	271	.613
3.82	140	.507	261	.456
4.52	136	.419	256	.371
	124		244	
	ρ = 46.0 per cent r_1 = 89.4 ohm cm.		ρ = 17.9 per cent r_1 = 291.0 ohm cm.	

If x_3 were due to a perfect dielectric capacity c_3

$$x_3 = s\,\gamma = \frac{1}{c_3\,\omega}$$

and the slope of the log s *vs.* log ω curve would be -1.0. This is in fair agreement with the data for red blood corpuscles but in no way

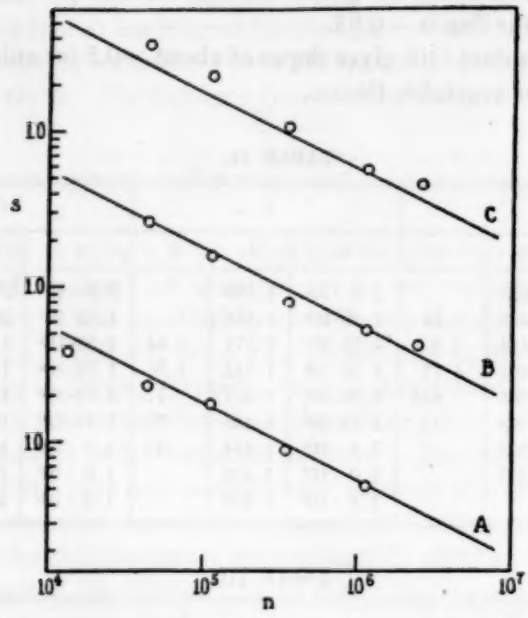

FIG. 5. Log s (Eq. 6) *vs.* log frequency (n, cycles per second) for *Arbacia* egg suspensions.

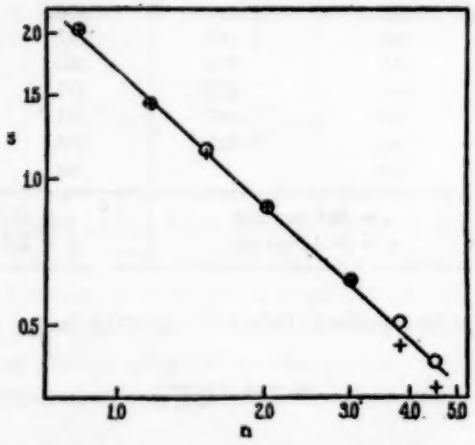

FIG. 6. Log s (Eq. 6) *vs.* log frequency (n, megacycles per second) for red blood corpuscle suspensions; data of Fricke and Morse.

agrees with that on tissues or on eggs—these giving capacities which vary inversely as the −0.25 and −0.5 power of the frequency. As stated in the previous paper, such variable capacities found in physical electrolytic systems have been classed as polarization capacities, and have associated with them an equivalent series polarization resistance. That the same is true for living systems is borne out by computations made on Gildemeister's data (4) for frog skin and on Blinks' unpublished data (1) for *Valonia*. For a single set of data for frog skin it was found that $m = 0.755$ and that the capacity varied as the −0.57 power of the frequency. For three sets of data it was found for *Valonia* that m was fairly constant for each case and varied from 0.63 to 0.75. Also the capacities varied with frequency[3] as a power having values from −0.13 to −0.23. From the biological point of view we should expect $r_3 > 0$, since it may be expected to be more or less proportional to the permeability for ions—being an equivalent *series* resistance. In such cases, the initial assumption that $r_3 = 0$ is not valid, and it is necessary to go to the more general expression, Eq. (10),[1] for $|z_3|$. When impedance data alone are available it is impossible to use this relation without the assumption that m of Eq. (13) is constant. Even then, no convenient method of computation has as yet been found. It has however, been possible to show empirically by the calculation of hypothetical data that as m is allowed to take on different values in different cases—but assumed constant in each case—,

$$\frac{|z_3|}{\gamma} = s^{\rho},$$

where ρ varies from one to two as m goes from zero to infinity. If

$$s = s_0\omega^q$$

where $s_0 = s$ when $\omega = 1$, then

$$\frac{|z_3|}{\gamma} = s^{\rho} = s_0^{\rho}\,\omega^{\rho q}.$$

It follows therefore that if we assume $r_3 > 0$, and consequently $m > 0$, we find that $|z_3|$ instead of varying as the −0.5 or −0.75 power of ω, will vary as a power of ω more nearly −1.0.

[3] *Cf.* Osterhout (8).

Since there is good reason to believe that $m \neq 0$, and no certainty that it is constant, unsupported impedance measurements have no immediate decisive value. It may still be interesting, however, to calculate what this capacity might be if it were a capacity alone. Since the measurements of volume concentrations have been so very unsatisfactory it is convenient to use an expression for γ which does not involve ρ explicitly. It can be shown that

$$\gamma = \frac{r_0^3 (2 + r_1/r_0)(1 - r_1/r_0)a}{2(r_0 - r_\infty)}.$$

This relation has been used in the calculation of x_3 in place of γ of Eq. (9) of the previous paper.[1] The values c_3 for n about $3.5 \cdot 10^6$ cycles per second are given in Table IV.

TABLE IV.

	r_0	r_∞	r_1	n	z	c_3
A	33.8	30.8	24.8	$3.48 \cdot 10^6$	.865	1.02 μf/cm.^{2}
B	37.8	33.7	25.6	3.59	.77	1.03
C	52.3	38.8	25.0	3.59	1.08	.86

$2a = 75 \cdot 10^{-4}$ cm.

If we extrapolate to 10^3 cycles per second on the assumption that c_3 varies inversely as the 0.5 power of the frequency, we find $c_3 = 18\ \mu f$/cm.^{2}

For red blood corpuscles Fricke found 0.8 μf/cm.,^{2} which he computed would be the capacity of a monomolecular layer of oil if the static capacity formula used was assumed to hold for such dimensions. The value of 18 μf/cm.^{2} would thus lead to a layer only one-twentieth of this thickness at 1000 cycles and it would become "thinner" still at lower frequencies. This line of reasoning suggests that the barrier to ions at the surface of the cell—if a dielectric—must be of less than atomic dimensions, and leads one to the interesting possibility that the restriction on ionic transfer may be due largely to repulsive and attractive electrostatic forces of absorbed ions at the cell surface.

Fertilization.

In almost every run it was noticed that before insemination the values of the impedance for any given frequency were quite variable, giving rise to similar variations in the average specific resistance of the egg at both the high and the low frequencies. Immediately upon fertilization however, these quantities became quite constant and did not change noticeably thereafter. While it is possible that the temperature control was not adequate, it is more probable that the variations were due to some change in the average condition of the eggs in the suspension. It has been suggested that the cause may lie in the differing states of maturation of the eggs taken from different ovaries or different parts of the same ovary, and that the initiation of development completely masked these prefertilization irregularities. As a result it can only be said that there was found no specific change in the conductivity of the interior of the egg, or the impedance of the surface which can be definitely ascribed to the membrane formation. Previous work by Gray (6) and McClendon (7) has shown that there is a change in the low frequency conductivity of a centrifuged mass of eggs upon fertilization.[4] It may very well be that the change is so small that it is beyond the limits of the present apparatus. It should, however, be pointed out that at low frequencies almost all of the current flows through the intercellular spaces since the surface of the egg offers a very high impedance to low frequency currents. Under these conditions a small change in the size of the eggs (5) or in the conductivity of the intercellular liquid would cause a considerable change in the conductivity of the centrifuged mass, as has been pointed out by others. At low frequencies, $|z_3|$ may be so large that—even when it varies considerably—Eq. (6) of the previous paper[1] should be true. While this equation cannot be expected to hold except for small values of ρ, it is worth noticing that if ρ is near unity, as it would be for a centrifuged mass of eggs, r_0 varies rapidly with both ρ and r_1.

[4] In conversation, Dr. Hugo Fricke said that he had found a change in the capacity of a frog egg upon fertilization but gave no further details. Dr. Sterne Morse stated, also in a conversation, that he had found a change in the capacity of a suspension of *Arbacia* eggs upon fertilization that he thought could be measured. He was working with a capacity bridge at 1000 cycles.

SUMMARY.

Apparatus has been designed and constructed for the measurement of the electric impedance of suspensions of *Arbacia* eggs in sea water to alternating currents of frequencies from one thousand to fifteen million cycles per second. This apparatus is simple, rugged, compact, accurate, and rapid.

The data lead to the conclusions that the specific resistance of the interior of the egg is about 90 ohm cm. or 3.6 times that of sea water, and that the impedance of the surface of the egg is probably similar to that of a "polarization capacity". The characteristics of this surface impedance can best be determined by measurements of the capacity and resistance of suspensions of eggs.

No specific change has been found in the interior resistance or the surface impedance which can be related either to membrane formation or to cell division.

The interest and assistance of Professor E. L. Chaffee and Professor W. J. Crozier have been very much appreciated. The author is indebted to Mr. P. S. Bauer for the use of his wavemeter and microscope.

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STUDIES ON PERMEABILITY OF MEMBRANES.

V. The Diffusion of Non-Electrolytes through the Dried Collodion Membrane.

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Several investigators have already studied the diffusion of non-electrolytes across membranes with very narrow types of pores (1–3). As their experiments were carried on with Traube's copper ferrocyanide membrane or with bag shaped dried collodion membranes there were certain mechanical difficulties due to delicacy of the membranes themselves which prevented any great extension of the investigations. In general, however, the experiments did tend to establish the fact that the diffusion rate of a substance was much more strongly dependent on molecular weight or more accurately molecular volume when a membrane was present than when one was not.

In the present investigation we have been able to use the exceedingly durable flat type of dried collodion membrane described in one of our earlier papers (4). We have restricted ourselves to a small number of substances, attempting to study each under the best conditions available. Acetone, urea, glycerine, and glucose were selected as best suited to our purposes because these substances can be obtained in pure form and because quantitative estimation by microanalytical procedures is possible.

Arrangement of Diffusion Experiments.

In all of the experiments the arrangement of apparatus was essentially the same. A moist chamber large enough to hold four or five membranes was used to contain the outside solution. The membranes placed in this were kept from resting on the bottom by a frame of glass rods. At the beginning of an experiment about 20 cc. of distilled water was placed inside of each glass bell supporting a membrane, a solution of the substance to be investigated in desired concentration

poured into the outside chamber, and the membranes immersed in the solution. Evaporation was prevented by a tightly fitting lid. After a suitable time the liquid inside the membranes was removed and the amount of substance diffused from the solution on the outside determined. A fresh quantity of water was immediately placed inside the membranes which were returned to the chamber at once, so that a new experiment was begun at the conclusion of each preceding one. Thus, when a stationary rate of diffusion had been reached, that is, when the diffusing substance had established a stationary gradient across the membrane, as little as possible was done to disturb the gradient, and a series of experiments conducted to determine as accurately as possible the rate of diffusion.

Due to causes which will be discussed later the establishment of a stationary diffusion rate was often exceedingly slow. After we had recognized this fact it became customary to wait several weeks, during which time the liquids were frequently changed, before beginning the analyses. A relatively close agreement between the results of successive experiments was taken as an index of the establishment of the stationary condition.

In working with urea and glucose it was possible to prepare an outside solution of the exact concentration desired. With acetone and glycerine, however, it proved more convenient to make up the solutions in approximately the concentration desired and then determine their exact strength by analysis during the course of the experiments. Especially was this necessary in the case of acetone. Acetone is extremely volatile and evaporates from solutions so rapidly that it was impractical to maintain a solution of any definite concentration. When working with acetone special care was taken to see that the lid of the chamber containing the solution fitted tightly. The exact strength of the solution was determined by analysis at the beginning and end of each experiment and the average figure taken as the concentration during the experiment. With other substances the depth of the solution in the chamber was from 2 to 3 cm. With acetone a depth of about 15 cm. was used so as to lessen the relative surface exposed for evaporation. Moreover the bells supporting the membranes were tightly stoppered so that no acetone might reach the inside solutions by way of vapor in the chamber. Even with these precautions a steady decrease in the concentration of the acetone solution could be observed but it was very slight during the course of any one experiment. From time to time an amount of acetone equivalent to that lost by evaporation was readded to the solution.

The first substances with which we worked were acetone and glycerine. These early experiments were conducted at room temperature, the slight effect of daily variations being neglected. Later an ice box, maintaining and recording a constant temperature of about 2°C., became available and was utilized for all the experiments with glucose and urea and for many further experiments with acetone and glycerine. Besides providing a constant temperature the coldness of the ice box was of material assistance in preventing decomposition of the organic substances by bacteria.

Methods of Analysis.

Acetone.—Acetone was determined iodimetrically in most cases with 0.01 M solutions of iodine and sodium thiosulfate. When titrating with solutions of such low titer it is especially important to remember that in alkaline solution the hypoiodite ion passes over easily to the iodate ion by dissolved oxygen. It was therefore necessary to make parallel blank determinations under accurately corresponding conditions and to correct for this blank. In some of the experiments the acetone had to be determined in the presence of glycerine. As even the purest glycerine we were able to obtain contained traces of substances combining with iodine, the acetone was transferred from the glycerine containing solution into the alkaline hypoiodite solution by the aeration method of Folin (5). The parallel blank consisted in replacing the glycerine-acetone solution by one of glycerine only. Fortunately the iodine-combining substances of the glycerine were non-volatile.

Urea.—The urea used showed a negative test for ammonia with Nessler's reagent. Exposed to the air, even at the temperature of the ice box, such a solution gave a positive reaction after several days but it could be shown that this was due not to decomposition of urea but to ammonia in the air. In hermetically tight bottles no Nessler reaction occurred after several weeks. In performing our experiments the outside solution was frequently changed. Moreover, in preparation for analysis the inside solution was evaporated almost to dryness on the steam bath at neutral reaction, a process sufficient to remove traces of ammonia. The nitrogen was then determined by the micro Kjeldahl method (Direct Nesslerization method of Koch and McMeekin (5)). Control analyses with known amounts of urea under the same conditions showed the efficiency of the method.

Glycerine.—Glycerine was determined by the reduction of a standard chromic acid solution. The method used was derived from the procedure described by Bang (6) for the determination of fat in blood. The chromic acid solution was carefully standardized against solutions of pure glycerine made to known strength by specific gravity readings. We found 1 cc. of 0.1 N chromic acid solution equivalent to 0.00713 millimols of glycerine. This factor is in substantial agreement with that given in an analogous method used in the arts for the determination of crude glycerine (7). Our procedure was as follows: The solution of approximately 25 cc. volume was evaporated on a steam bath. We were able to show that evaporation caused no loss of glycerine provided it was not continued beyond a volume of 0.5 to 1 cc. Complete evaporation of the water resulted in losses of 10 per cent or more of the total glycerine. A sufficient quantity of 0.1 N chromic acid (usually not more than 2.0 cc.) was run in from a burette and 5 cc. of concentrated sulfuric acid added for each 2 cc. of chromic acid taken. When insufficient chromic acid had been used as shown by the change in color more was added and correspondingly more sulfuric acid. Complete oxidation of glycerine occurs within several minutes but usually we waited an hour before washing the

TABLE I.

Date	Membrane															
	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9	F-10	F-11	C-1	C-2	C-6	C-7	I-1
	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.
June, 1927	42.2	28.1	45.4	38.3	36.0	15.4	29.7	47.0	41.1	38.5	32.1	49.0	47.1	41.1	47.0	
July, 1927	41.8	26.3	45.4	38.2	33.7	16.3								40.7	48.3	
Sept., 1927	39.2	24.2	40.4	32.6	28.2	8.9	30.1	46.3	41.5	39.0	35.5	49.5	44.8	38.0	47.1	
Dec., 1927	35.7	22.2	35.6	28.1	23.2	12.3	25.8	40.3	36.1	34.2	25.7	49.7	46.8	35.9	45.4	49.6
Feb., 1928						12.5						49.4			47.5	48.0

Shows potential differences between 0.1 N and 0.01 N KCl solutions (*Co P*) with the membranes used in this investigation at various times during the period of study. These *Co P* readings have been taken as an index of the degree to which the initial properties of the membranes were maintained.

solution into a large beaker, adding several cubic centimeters of 5 per cent potassium iodide solution, and titrating the liberated iodine with 0.1 N sodium thiosulfate solution.

Glucose.—Glucose was determined by the method of Hagedorn and Jensen (5) used for blood, omitting the steps concerned with the removal of protein.

Constancy of the Membranes.

The reproducibility of the potential difference given by a membrane between 0.1 N and 0.01 N potassium chloride solutions (*Co P*) was taken as a gauge of the constancy of the properties of the membrane. In Table I we have arranged the *Co P* values of the membranes used in this study at the beginning of the experiments and subsequent readings made from time to time during the period. The method of making these readings has been previously described. In general it required a week before a constancy of potential within a millivolt was reached. After this time several readings were made on successive days and the average figure entered as the correct *Co P* value. It will be seen that in most of the membranes a drop of several millivolts occurred during a period of 6 months while in a few the *Co P* value remained essentially unchanged. It may be that some of the minor discrepancies to be reported later may be attributed to these slight changes in the membrane properties.

Among the substances with which we worked there is one, acetone, which in high concentration can act as a solvent for collodion. One can suppose that in more dilute solutions a trace of this action might remain, that the acetone might cause the collodion to swell and alter somewhat the properties of the membrane. In other words it might be suspected that in this particular case the membrane might function not only as a sieve but also as a solvent and so alter the mechanism of diffusion. In the concentrations actually used in our experiments, in only a few cases greater than 0.1 N or about 0.6 per cent, the property of acetone as a solvent for collodion could neither be expected nor observed. However, in order to establish the insignificance of acetone in these low concentrations on the membrane properties *Co P* measurements were made with one membrane in which the two potassium chloride solutions on the two sides of the membrane contained acetone in 0.1 M concentration. The ordinarily obtained value

for the *Co P* of this membrane was 50 millivolts. In the presence of acetone it was 48 millivolts. The original value was reobtained when the acetone-containing potassium chloride solutions were replaced by pure potassium chloride solutions. The change was very slight and reversible. Without attempting a definite explanation for the slight change observed it may be mentioned that the effect of acetone on the activity coefficient of potassium chloride at different concentrations is quite sufficient to account for the small difference.

Selection of Form for Expressing Results.

If the ordinary rules of diffusion are applicable the amount of a substance which will diffuse through a membrane is proportional to the time, to the exposed area, to the difference in concentration existing between the solutions at the two borders of the membrane, and inversely to the thickness of the membrane or length of the pore channels. The constant which converts this proportionality into an equation is called the diffusion coefficient. In these investigations we are to deal with a comparison of the rates at which two different substances diffuse through the same membrane and in making such a comparison may regard the exposed area and membrane thickness as constant. Moreover, the time of diffusion was never long enough to alter appreciably the difference in concentration between the liquids on the two sides of the membrane and this difference may likewise be considered a constant. For this reason it will be sufficient for our purposes to express the rate of diffusion for each membrane with each substance in a definite concentration in millimols passing through the membrane per hour. Inasmuch as we are presently to demonstrate that the rate of diffusion with our membranes is proportional to the difference in concentration, that is, that this principle of Fick's law holds also for the dried collodion membrane, it will be possible to express all diffusion rates in terms of the rate that would hold for some fixed reference concentration. For convenience throughout this paper we shall express our results in terms of the diffusion rate holding for 0.1 M solutions.

Concerning the effect of temperature it may be stated that the factor of dependence is not large and that in view of other experi-

mental errors incident to these experiments a variation of 1° or 2°C. is of negligible significance. In the experiments conducted in the ice box a constant temperature was maintained.

Dependence of Diffusion Rate on Difference in Concentration between Solutions on Two Sides of Membrane.

Our first problem was to show that the rate of diffusion for any membrane was proportional to the difference in concentration between the two solutions on the two sides of the membrane. We did not feel that we would be justified in speaking of a diffusion coefficient in the true sense of Fick's law unless such a relation could be shown to exist. Furthermore it was evident that much economy of time would result if we were able to conduct experiments with the more slowly diffusing substances in higher concentrations and if all results could then be referred to a common unit.

The first suggestive proof that such a definite relation between concentration and rate of diffusion did exist was furnished by a few experiments with glycerine in 0.5 M and 0.1 M concentrations. Four different membranes were used. The result is shown in Table II. The average diffusion rate for the four membranes was 2.00×10^{-5} millimols per hour for the experiments in 0.5 M concentration when expressed in terms of a supposedly proportional rate for 0.1 M concentration and 2.20×10^{-5} millimols per hour for the experiments actually conducted at this concentration. The agreement is as good as can be expected when one considers the small amounts obtainable for analysis at the lower concentration.

In another series of experiments with acetone done at the same time the diffusion coefficients obtained from the experiments in 0.5 M concentration and those in 0.1 M concentration were essentially the same but the experiments in more dilute solutions led to higher relative rates. We surmised that this discrepancy was the result of experimental errors rather than real and accordingly planned another series of experiments with acetone in which the region of concentration which had previously shown the discrepancy was specially investigated. In this series care was taken to eliminate the supposed sources of error in the first series. A period of 3 weeks was allowed for the establishment of the stationary diffusion gradient in the more dilute of the

solutions before any analyses were made. A constant temperature was maintained in the ice box. The results of this series are shown in Table III. Although the rates as determined from individual experiments, especially those in 0.01 M concentration, vary considerably, a comparison of the average rates from each group of experiments shows a striking uniformity. The average of the four membranes shows a rate of 28.9 $\times$ 10^{-5} millimols per hour determined when the concentration of the outside solution was 0.1 M and a relative rate of 29.6 $\times$ 10^{-5} millimols per hour calculated from the experiments in 0.01 M concentration.

It is necessary at this point to call attention to the rather large discrepancies occurring between the results of individual experiments. These discrepancies are far beyond the limits of error of the analytical

TABLE II.

Membrane	F-3	F-5	F-6	C-7
Diffusion rate observed in 0.5 N concentration.	8.04	3.94	15.45	12.45
0.1 N diffusion rate calculated from above....	1.61	0.79	3.09	2.49
Observed 0.1 N diffusion rate.....	1.56	0.77	3.56	3.02

Experiments with glycerine indicating that the diffusion rate is proportional to the difference in concentration between the inside and outside solutions. All rates are expressed in terms of millimols $\times$ 10^{-5} per hour.

methods employed. They were observed throughout all the experiments of this study,—with urea, glycerine, and glucose as well as with acetone. Moreover, it will be observed that with some membranes the tendency to show variations in this respect is much greater than in others in which the results of individual experiments are fairly uniform. In the experiments in 0.01 M concentration listed in Table III the results with the membrane F-7 show an average deviation from the mean rate of 30.9 per cent, while in the experiments with the membrane F-9 the average deviation is only 5.4 per cent. These considerations have led us to believe that the tendency to discrepancy between the results of individual diffusion experiments is an inherent property of the membrane itself. An explanation of this phenomenon will be attempted later. For the present it is sufficient to call attention to its existence as an explanation for the adopted method of ob-

TABLE III.

Membrane	Rate calculated from experiments in 0.01 M concentration	Average rate from 0.01 M experiments	Rate calculated from experiments in 0.1 M concentration	Average rate from 0.1 M experiments
F-7	19.9	39.5	35.1	34.1
	21.7		31.8	
	51.0		35.4	
	46.6			
	34.3			
	54.2			
	48.8			
F-8	25.8	21.4	22.0	21.9
	20.6		22.9	
	21.9		20.8	
	15.2			
	22.3			
	20.2			
	23.9			
F-9	37.9	35.5	37.4	38.2
	34.0		40.8	
	39.9		37.0	
	32.3		37.5	
	33.7			
	35.4			
	35.6			
F-10	33.5	21.8	20.3	21.4
	18.9		24.2	
	22.7		21.2	
	17.4		20.0	
	20.9			
	17.4			

Second series of experiments with acetone to show the relation between difference in concentration on the two sides of the membrane and rate of diffusion. Results of individual experiments are given together with average results for each series of experiments. All experiments performed at constant temperature of ice box. All rates are expressed in terms of a supposedly proportional 0.1 M rate and as millimols $\times 10^{-5}$ per hour.

TABLE IV.

Membrane	Rate of diffusion of acetone	Average acetone rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates $\frac{\text{acetone rate}}{\text{glycerine rate}}$
F-1	104.0				
	122.8				
	133.1		1.95		
	116.7	123.0	1.80	1.71	71.9
	124.7		1.44		
	130.5		1.66		
	117.6				
	134.2				
F-2	80.2				
	78.7				
	83.6		1.60		
	100.2	83.2	1.58	1.50	55.5
	69.0		1.53		
	102.1		1.28		
	68.1		1.50		
	83.9				
F-3	140.2				
	104.4				
	113.9				
	144.5		1.82		
	141.6		1.40		
	156.0	125.3	1.45	1.56	80.3
	101.5				
	109.1				
	116.7				
	136.1				
	114.8				
F-4	75.9				
	97.7		1.84		
	89.1		1.75		
	99.5	92.1	1.60	1.66	55.5
	73.4		1.60		
	102.9		1.49		
	94.6				
	104.0				

Shows two series of experiments in which rate of diffusion was determined with both acetone and glycerine. Last column indicates the ratio of these rates. Majority of acetone experiments were conducted in a concentration of approximately 0.1 M; majority of glycerine experiments in a concentration of approximately 0.5 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at room temperature.

TABLE IV—*Concluded.*

Membrane	Rate of diffusion of acetone	Average acetone rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates $\frac{\text{acetone rate}}{\text{glycerine rate}}$
F-5	78.8				
	63.9				
	80.2		0.68		
	81.8		0.89		
	86.7	76.8	0.73	0.78	98.5
	81.3		0.80		
	60.9				
	56.7				
	95.0				
	82.6				
F-6	79.6				
	58.2				
	71.1		2.92		
	70.3		3.26		
	79.6	77.6	4.69	3.33	23.3
	75.3		2.43		
	76.7				
	79.4				
	105.0				
	80.8				
C-6	181.8				
	190.6				
	134.9		7.47		
	160.7		7.21		
	120.9		7.85		
	120.7	167.4	6.92	7.04	23.8
	188.9		6.99		
	163.8		5.77		
	160.1				
	221.7				
	203.6				
	161.6				
C-7	237.0				
	251.3				
	318.5		2.49		
	305.7		2.11		
	314.0		3.92		
	258.2	243.5		2.84	85.7
	183.1				
	213.4				
	187.9				
	216.9				
	193.0				

taining the diffusion rate in any particular case from the mean result of a series of experiments rather than from a single experiment.

TABLE V.

Membrane	Average diffusion rate of acetone	Rate of diffusion of urea	Average urea rate	Ratio of rates $\frac{\text{acetone rate}}{\text{urea rate}}$
F-7	36.8	16.3	13.6	2.71
		12.5		
		13.7		
		14.8		
		10.8		
F-8	21.7	17.5	17.2	1.26
		14.6		
		16.3		
		24.1		
		13.7		
F-9	36.9	29.4	28.0	1.32
		25.8		
		27.0		
		32.1		
		25.7		
F-10	21.6	18.1	16.8	1.29
		13.8		
		18.0		
		21.9		
		12.4		

Shows two series of experiments in which the rate of diffusion was determined with both acetone and urea. With urea the results of individual experiments and the average result are given. With acetone only the average result is given; the results of the individual experiments were given in Table III. Urea experiments were conducted in a concentration of 0.1 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at constant temperature of ice box.

Experiments to Determine Relative Rates at Which Various Non-Electrolytes Diffuse through the Dried Collodion Membrane.

In these experiments we did not follow the method used by Fujita in which mixtures of two different substances were placed in solution

on one side of the membrane and distilled water on the other side and the relative quantities of the two substances diffusing through the membrane determined. Because of the durability of our membranes we were able to work with one substance at a time, measuring as accurately as possible by a series of experiments the diffusion rate for each substance. We then compared the rates at which the different

TABLE VI.

Membrane	Rate of diffusion of urea	Average urea rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates $\frac{\text{urea rate}}{\text{glycerine rate}}$
C-1	644.1	597.6	26.2	28.0	21.3
	552.4		31.2		
	596.4		29.3		
			25.4		
C-2	570.8	546.4	84.9	92.4	5.9
	511.8		100.6		
	556.5		91.8		
F-11	16.7	13.8	0.54	0.52	26.5
	10.4		0.52		
	14.3		0.51		

Shows two series of experiments in which the rate of diffusion was determined with both urea and glycerine. Last column indicates the ratio of these rates. Urea experiments were conducted in a concentration of 0.1 M; glycerine experiments in a concentration of approximately 0.5 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at constant temperature of ice box.

substances diffused. By this method we eliminated the possibility of changes in the diffusion rates due to the mutual action of two substances on each other.

Acetone-Glycerine.—The results of experiments in which the diffusion rates for acetone and glycerine were compared have been listed in Table IV. It will be seen that with the membranes used the ratio of the diffusion rates varied between 23 to 1 and 98 to 1, acetone being the more rapid. This difference is far beyond any sources of error. Even with the membrane giving the smallest figure the ratio is many times larger than would be expected if no membrane were present.

With no membrane, that is in free aqueous diffusion, the rates vary approximately inversely with the square root of the molecular weight. In this case the ratio of the rates would be 1.26 to 1.

Acetone-Urea.—In Table V have been listed the results of experiments comparing the diffusion rates of acetone and urea. It will be seen that acetone diffuses 1.3 to 2.7 times as fast as urea. The molecular weight of urea (60) is slightly greater than that of acetone (58). We cannot insist that this slight difference is sufficient to account for the difference in the diffusion rates for an accurate comparison would

TABLE VII.

	Membrane					
	C-1	C-6	C-7	F-2	F-8	F-11
Diffusion rates from individual experiments	0.83	1.28	0.17	0.49	0.58	0.37
	0.61	2.00	0.22	0.56	0.66	0.43
	0.42	1.36	0.28	0.60	0.52	0.36
	0.46	1.22	0.17	0.89	0.49	0.29
	0.55	1.65	0.20	0.65	0.43	0.33
	0.44	1.70	0.22	0.67	0.38	0.35
	0.51	1.79	0.22	0.83	0.67	
	0.87				0.70	
	0.85				0.77	
					0.33	
					0.17	
					0.24	
					0.25	
Average rate.....	0.616	1.57	0.211	0.670	0.476	0.355

Results of diffusion experiments with glucose. Performed at ice box temperature and with outside solution of 0.5 M concentration. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour.

have to depend on a knowledge of the molecular volume, water envelope and intermolecular forces, and these only approximately depend on the molecular weight. We shall merely state that these two substances with almost the same molecular weights have also approximately the same diffusion rates.

Urea-Glycerine.—In addition to the experiments with acetone and glycerine and those with acetone and urea which in themselves permit us to form an estimate of the urea-glycerine diffusion ratio, a few

experiments were performed in which the diffusion rates of these two substances were directly compared These have been listed in Table VI. It will be seen that urea diffused 6 to 26 times as rapidly as glycerine.

Glucose.—No separate set of experiments was done in which the diffusion rate of glucose was compared with one other substance.

TABLE VIII.

Membrane	Average acetone rate in pure solution	Rate of diffusion of acetone in presence of glycerine	Average acetone rate in presence of glycerine
F-3	125.3	120.3	130.3
		127.6	
		143.1	
F-5	76.8	66.1	79.5
		82.1	
		90.2	
F-6	77.6	63.0	65.2
		59.9	
		72.7	
C-7	243.5	231.4	262.4
		280.7	
		206.6	
		330.7	

Shows results of two series of experiments in which the rate of diffusion of acetone was determined. In the first series (given in detail in Table (IV) pure water was placed inside the membranes and approximately 0.1 M acetone solution outside. In the second series the concentration of the outside solution remained 0.1 M with respect to acetone but in addition both inside and outside solutions were 0.1 M with respect to glycerine. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at room temperature.

Rather we selected membranes used in previous experiments with which the diffusion rates of two other substances were already known. The results of the individual experiments are listed in Table VII. By comparing the diffusion rates of glucose with the rates for other substances given in the previous tables, it will be seen that the rate with glucose was always much smaller than with any of the other sub-

stances studied. Later we will list and discuss the ratios of the glucose rates to each of the other substances.

Experiments to Determine the Effect of One Substance on the Diffusion Rate of Another.

Inasmuch as in his experiments Fujita (2) had worked with mixtures of two substances we thought it worth while to find out whether the presence of one substance would change the diffusion rate of another. It was conceivable that some of the membrane pores might become clogged with the molecules of one substance and that fewer pores would be available for the diffusion of the other. For this purpose we determined to see whether the rate of diffusion of acetone could be altered by the presence of glycerine. Four membranes were selected in which the rate of diffusion of acetone was accurately known. These membranes were allowed to stand for several weeks in contact on both sides with a 0.1 M solution of glycerine. Then enough acetone was added to the outside solution to make it 0.1 M with respect to acetone as well as glycerine, and after a suitable time allowed for the establishment of a definite diffusion gradient, the rate at which the acetone diffused through the membranes in the presence of glycerine was measured. The results have been arranged in Table VIII. It will be observed that we were unable to detect any effect of the glycerine on the rate of diffusion of acetone.

Experiments to Indicate Steps Involved in Establishment of Stationary Diffusion Gradient.

It has already been pointed out that in these diffusion experiments we found it necessary to wait some days for the establishment of a definite diffusion gradient before any consistancy of results could be obtained. It seemed highly desirable that we should have some idea of the processes going on during the establishment of this definite gradient. With the substances with which we first worked only rough ideas could be obtained as the analytical methods available were not sufficiently sensitive to determine quantitatively the small amounts diffusing in the first few hours. With acetone we did observe that in general the result obtained from a first or second experiment

was lower than later ones. However, when we began to work with the extremely accurate micro methods available for the determination of sugar it became possible to make such studies quantitatively even though the diffusion coefficient of sugar is many times smaller than that of acetone. Experiments were begun with membranes which had been kept in water for several weeks previously and which had never been in contact with sugar. A 0.5 M glucose solution was used. From the first we determined at regular intervals the quantity of sugar passing the membrane. From these figures it was possible to

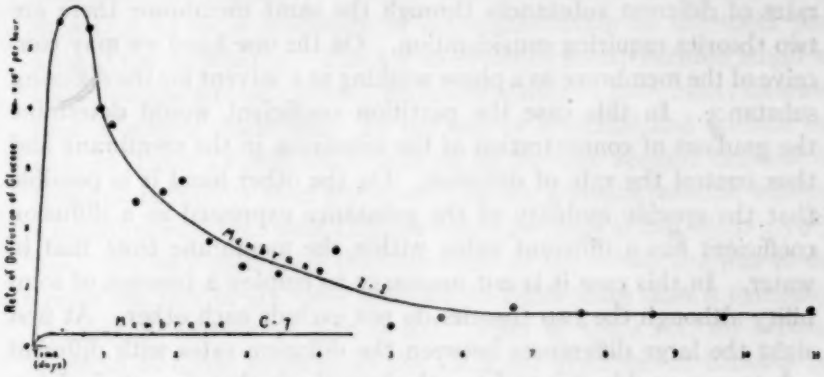

CHART 1. Shows the various diffusion rates holding during the period of establishment of the stationary diffusion gradient of glucose with two different membranes. The dots represent the results of individual experiments, each dot being charted in the middle of the time period over which the diffusion was measured. The curves have been drawn by interpolation.

construct a curve showing the changes in diffusion rate during the establishment of the stationary gradient. In Chart 1 we have drawn two curves illustrative of two different types of processes involved. In the experiments with the membrane I-1 the most rapid rate was obtained from the first experiment. After this the rate decreased from day to day reaching a stationary value at about the 6th day. We have drawn the first part of the curve beginning at zero because it is evident that some time must have elapsed before any glucose could have crossed the membrane. The impression gathered from this curve is that the process is similar to the one observed when a protein

solution is filtered through an ultrafilter,—the pores of the filter gradually becoming clogged. Here it would seem as though the glucose molecules had gradually clogged or plugged up many of the channels in the membrane which were available at first. In contrast to this curve stands the one obtained with the membrane C-7. Here the diffusion rate was quite constant from the beginning.

DISCUSSION.

In offering an explanation for the large differences in the diffusion rates of different substances through the same membrane there are two theories requiring consideration. On the one hand we may conceive of the membrane as a phase working as a solvent for the diffusing substance. In this case the partition coefficient would determine the gradient of concentration of the substance in the membrane and thus control the rate of diffusion. On the other hand it is possible that the specific mobility of the substance expressed as a diffusion coefficient has a different value within the membrane from that in water. In this case it is not necessary to employ a concept of solubility although the two theories do not exclude each other. At first sight the large differences between the diffusion rates with different substances would seem to favor the hypothesis that the rate is determined in the greater part by solubility. This is because we are accustomed to large differences in solubility between different substances but not to large differences in mobility. For this reason Northrop (8) recently gave preference to the solubility theory. In previous papers we have treated the dried collodion membrane as a sieve membrane with pores almost of molecular size. When the membrane channels are as minute as this a distinction between a molecule in solution in the membrane and one within a channel becomes doubtful. A full discussion of this problem of greatest importance to an understanding of membrane action would lead us far from the purpose of this paper. In a later communication we shall bring together a number of facts which enable us to give preference to the theory of sieve action and during the present paper will accept this theory without further discussion.

In attempting then to form a mental picture of the mechanism of diffusion in our experiments which would account for the observations

made two possibilities were kept in mind. We thought it probable that the great difference in the diffusion rates between molecules of different sizes was due to the fact that the membranes contained pore channels of varying sizes, that only the largest of these channels would serve for the passage of a large molecule such as glucose, and that many more channels could be utilized by the smaller molecules such as acetone and urea. Now it seems rational to suppose that the quantity of any of these substances which can diffuse in a given time will be proportional to the total area of all the pores large enough to function in its transport. This area may be called the available pore area (A.P.A.) for a substance in contradistinction to the total pore area of the membrane. The A.P.A. evidently must become larger as the molecular size decreases. An example may be more easily followed than a general discussion. In the case of glycerine and acetone the data in Table IV will show that on an average acetone diffused 62 times more rapidly than glycerine. In free aqueous solution without a membrane acetone would diffuse only 1.26 times as rapidly as glycerine. We may then state that the effect of the membrane was to decrease the rate of glycerine diffusion 49 times more than it decreased the acetone rate ($49 \times 1.26 = 62$). That is, the A.P.A. for acetone must be 49 times as great as the A.P.A. for glycerine or because the former includes the latter as one of its parts it follows that only 2 per cent of the A.P.A. for acetone is available for glycerine. Similarly it can be shown by analysis of the figures in which glycerine and glucose were compared that only 10 per cent of the A.P.A. for glycerine was available for glucose and from the experiments with acetone and glucose that only 0.3 per cent of the A.P.A. for acetone could be utilized by glucose. Such differences as these can exist only if the number of pores utilizable by the substances of smaller molecular size are many times greater than the number of larger pores and we may conclude that in general as the size of the pore channels increases the number of these channels greatly decreases.

However it is possible to formulate another hypothesis in which the great difference with which molecules of larger and smaller size diffuse may be attributed to increased frictional resistance of the larger molecules against the pore walls rather than to variations of pore size. The experiments just described have led us to believe that this explana-

tion is not the major one. We have seen that a membrane saturated with glycerine has essentially the same permeability to acetone as in the absence of glycerine. If the same membrane channels were being utilized for the passage of glycerine as for acetone it is hard to believe that the more slowly moving glycerine molecules would not greatly interfere with the diffusion of acetone. On the other hand, if the first hypothesis is the correct one, the smaller pores cannot be utilized by glycerine and their channels will remain open even in the presence of a high concentration of this substance and can function for the transport of acetone. As the pore area that can be entered and clogged by the glycerine molecule is only 2 per cent of the A.P.A. for acetone it follows that 98 per cent should still be able to function in carrying acetone. Because of this it is scarcely to be expected that we would be able to detect any effect on the rate of diffusion of acetone and this is in accordance with the observations.

Again we have called attention to the fact that with some membranes during the period of establishment of the stationary diffusion gradient the rate at which glucose diffuses is more rapid during the first days or hours than later when the rate has become constant. It is not possible to account for this observation on the assumption that friction alone is responsible for the slow rate of diffusion of glycerine. We have explained it upon the basis of the first hypothesis by supposing that some of the membrane channels are of such a size that they are permeable to glucose for a short time only. Gradually as the diffusion continues they become clogged and the final constant diffusion rate is maintained by pores of slightly larger size which do not become clogged. We are conscious of the fact that the statement that a membrane channel has been clogged with a molecule may not mean that the movement of this molecule has been stopped entirely but merely that its progress has been hindered for a time and that such a conception would be equivalent to admitting that friction does play a part. However, we could not on this basis, account for the regular way in which the diffusion rate decreased from day to day until a stationary rate was reached. It is possible, and we believe probable, that the final stationary rate represents a condition not only in which some pores have remained permanently open and some permanently closed but also in which another group of pores are being

temporarily occluded and then reopened in such a manner that more or less of an equilibrium is maintained. We are inclined to believe that the smaller irregularities in the diffusion rates found from day to day not only with glucose but also with the other substances are due to the unevenness of this process.

In summarizing, then, the points just discussed which have helped us form a picture of the structure of our membranes, we may conclude that the pore channels are of many different sizes. We know nothing of the number or importance of the pores smaller than the acetone molecule but have been able to estimate that of the remaining pore area only 2 per cent is distributed among pores large enough to allow the diffusion of glycerine and only 0.3 per cent among pores large enough for glucose.

In previous papers of this series (4, 9) we have dealt with the electrical effects demonstrable when the dried collodion membrane is placed between solutions of the same electrolyte in different concentrations. The *Co P* value when a membrane is placed between 0.1 N and 0.01 N KCl solutions is the most familiar example. In a general way we have taken this *Co P* value as a criterion of certain of the membrane properties and in particular have regarded it as giving some sort of an average estimate of the fineness of the pore channels. It would seem then that we should be able to draw more or less of a parallel between the *Co P* values of our membranes and the ratios of the diffusion rates between molecules of different sizes, for we have regarded these ratios as depending upon the relative numbers of pores of larger and smaller size. In the early experiments of this study with acetone and glycerine we were disappointed in being able to find no such expected parallel. It is true that the membrane with the lowest *Co P* value, F-6, also gave the lowest acetone-glycerine ratio but another membrane, C-6, with a moderately high *Co P*, showed almost the same ratio. Similarly the membrane F-5, with a *Co P* value considerably below the average, gave the highest acetone-glycerine ratio of all. However, when one examines the figures secured from the experiments with glucose quite a different result is obtained. The glycerine-glucose, acetone-glucose, and for comparison the acetone-glycerine ratios with this series of membranes have been listed in Table IX, the membranes being arranged in order of

their *Co P* values. From this table it can be seen that no relation exists between *Co P* and the acetone-glycerine ratio,—that between the acetone-glucose ratio and *Co P* a suggestive relation exists in that only the two membranes with the highest and best maintained *Co P* values gave the very large ratios,—whereas between the glycerine-glucose ratio and *Co P* values with one exception a definite parallel can be drawn, the ratios increasing directly with the *Co P*. These ratios increase in a regular way from 1.46 for the membrane F-11 with

TABLE IX.

Membrane No.	*CoP* variations	Ratios of Diffusion Rates		
		Glycerine / Glucose	Acetone / Glucose	Acetone / Glycerine
	mv.			
F-2	28.1–22.2	2.09	115.8	55.4
F-11	32.1–25.7	1.46	64.2	43.8
C-6	41.1–35.9	4.18	99.4	23.8
F-8	47.0–40.3		45.4	
C-7	47.0–45.4	12.56	1075.8	85.7
C-1	49.7–49.0	45.45	1600.6	35.2

To show relation of *Co P* to various diffusion rate ratios. In this table the membranes studied with glucose have been arranged in order of their *Co P* values which are given with their variations during the period of study. In preparing the table several approximations were necessary. With the membranes C-1 and F-11 the acetone diffusion rate was calculated from the urea diffusion rate by multiplying the latter by 1.65 (average value for known acetone-urea ratio). With the membranes F-2, C-6, and C-7 the acetone and glycerine diffusion rates were determined at room temperature (about 23°C.); for comparison with glucose these rates were corrected for a temperature difference of 20°C. between the room and ice box by supposing the diffusion rates proportional to absolute temperature.

an average *Co P* of 28.9 millivolts to 45.45 for the membrane C-1 with an average *Co P* of 49.4 millivolts.

In order to harmonize and account for these apparently diverse results it is necessary to turn to a consideration of those factors in the membrane which are responsible for the potential difference arising when it is placed between solutions of an electrolyte in different concentrations. We have previously shown that this potential difference is dependent not only upon the ratio of the concentrations

of the electrolyte but also upon the particular concentration range being considered. In dilute solutions the effect of the membrane is marked and relatively high potential differences are observed whereas in more concentrated solutions the effect of the membrane tends to vanish. Moreover, we have shown that these effects can be satisfactorily explained by referring them to changes in the transfer number of the anion or more directly to changes in the mobility of the anion produced by the membrane. Thus when working with potassium chloride in high concentration the mobility of chlorine is almost as great as that of potassium and the observed potential effect is small. In low concentration the mobility of chlorine is small compared with that of potassium and a relatively large potential effect arises.

It now becomes of interest to search more closely for the factor responsible for the changes in the mobility of the anion with changes in concentration. It will be recalled that in any case the mobility is much less in the membrane than in free aqueous solution. We may suppose that this decrease in mobility is due to some sort of adhesion or adsorption between the anions and the boundary surface of the pore channels. When the concentration of the electrolyte is low enough there may be a channel surface great enough to bind all or nearly all of the anions migrating through the membrane. Similarly in a high concentration the channel surface may be great enough to bind only a small part of the migrating anions, the remainder moving with relative freedom through the channel lumens. We may thus refer the changes in the mobility of the anion to changes in the ratio between the number of anions in the solution and the area of the channel walls. With any membrane we can vary the first of these factors by changing the concentration of the electrolyte in the solution wetting it. The effect of varying the area of the channel walls cannot be observed in one membrane because this area is fixed. However, we can see in this factor an explanation for the fact that one membrane in a high range of concentrations may give the same potential difference as another membrane in a low range. Theoretically it is possible to conceive of a membrane with a constant pore-lumen area showing widely different potential effects depending upon whether

this area is made up of a few pores of relatively large size or many smaller pores.

In a previous paper we have shown that the curve expressing the dependence of the transfer number of the anion on concentration has a bilevel form.* In high and in low concentrations the dependence is slight; in a medium concentration range the dependence is great. Inasmuch as this bilevel curve is brought about when we vary in a regular way the ratio between the number of anions and the area of the channel walls by varying the concentration of the electrolyte we may infer that if we could vary in a regular way the size of the pore channels, the resulting curve expressing the transfer number of the anion would likewise have a bilevel shape for the effect on the determining ratio would be the same as produced by concentration changes. Thus we may suppose that there is a certain range of pore sizes through which the potential effects will vary widely. Pores of smaller size than those included in the range will have little more effect than if they were large enough to be included in the smaller end of the range, and similarly the effect of increasing the size of a pore will be slight after this range is surpassed.

Now, in the case of non-electrolytes diffusing through the membrane, there are no adhesive forces from the cell walls such as are present with an electrically charged ion. The rate of passage depends merely upon the number of pores large enough to transmit them. We have here then a rational explanation for the seemingly diverse results obtained in our diffusion experiments. We may imagine that the majority of the pores which allow the passage of acetone and glycerine are too small to be included in the range responsible for marked potential variations. The ratio of the glycerine rate to the acetone rate would then tell us nothing of the electrical effect to be expected. On

* As curves of this shape are encountered very often in biochemical investigations (dissociation curves, titration curves, etc.) we have introduced the descriptive term "bilevel" by which they may be designated. Such a curve may be defined as one in which the dependent variable depends on the independent to an appreciable extent only within a limited range beyond which the curve flattens on both sides to two parallel asymptotes each at a different level. Moreover, the curve has only one inflection point which lies in the variable part of the curve and the sign of the first derivative never changes. Such a curve is represented in Chart 2.

the other hand it is extremely likely that the pore sizes between the size of the glycerine and glucose molecules include at least a part of this range for here there is an evident relation between the diffusion rate ratios and the *Co P* values.

It may be helpful to represent the conception embodied in the foregoing discussion in a schematic way (Chart 2). Here we have set S_1, S_2, S_3, etc. to stand for the pores of increasing size in a membrane and N_1, N_2, N_3, to represent the number of pores of each respective size. Then the entire pore system of the membrane can be represented by $N_1S_1 + N_2S_2 + N_3S_3$, etc. The curve shows the manner in which

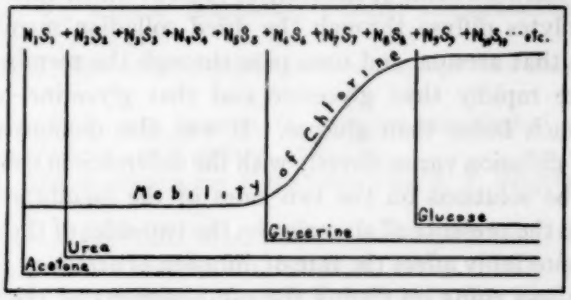

CHART 2. Schematic representation of the pore system of the dried collodion membrane. S_1, S_2, S_3, etc. refer to different and increasing pore sizes; N_1, N_2, N_3, etc. indicate the number of pores of each size. The bilevel curve shows an hypothetical zone in which anion mobility varies greatly with increasing pore size. The possible pore sizes concerned in the diffusion of the various non-electrolytes are indicated in the lower part of the diagram.

the mobility of the anion and therefore the *Co P* value may depend upon the various pore sizes. The lower part of the chart shows the pore sizes able to function in the diffusion of acetone, urea, glycerine, and glucose. An inspection of this chart shows that countless arrangements of pore sizes and numbers are possible but that only when these fall within a certain range will there be any effect on the *Co P*.

We are aware that the foregoing theory offers no explanation for the fact that the acetone-glucose ratio does not correspond with the *Co P* value as well as the glycerine-glucose ratio. Referring again to Chart 2 it will be seen that both of these ratios cover the pore size

range of electrical importance. As yet we do not feel prepared to offer an adequate explanation for this discrepancy. We may state, however, that several other considerations have rendered plausible the idea that in a certain range of pore sizes there may be an effect on cation mobility as well as anion mobility and that by a development of this idea it is possible to account for the discrepancy just mentioned. However, we shall not feel justified in developing this idea before definite experimental data on this point has been collected.

SUMMARY.

A study has been made of the relative rates at which various organic non-electrolytes diffuse through the dried collodion membrane. It was found that acetone and urea pass through the membrane many times more rapidly than glycerine and that glycerine in its turn diffuses much faster than glucose. It was also demonstrated that the rate of diffusion varies directly with the difference in concentration between the solutions on the two sides of the membrane. It was shown that the presence of glycerine on the two sides of the membrane did not appreciably affect the rate of diffusion of acetone. In a study of the changes going on during the establishment of the stationary diffusion gradient with glucose experiments were described which strongly suggested that many of the membrane channels may gradually become clogged up with glucose molecules so that the diffusion rate decreases from day to day until the stationary gradient is finally reached.

In explaining the various experimental data the conception of the collodion membrane as a sieve with pores approximating in smallness the size of individual molecules was utilized. The large differences in the diffusion rates between different substances were then referred to differences in molecular size, the relatively large molecules of glycerine and glucose being unable to pass through many of the smaller pores available for urea and acetone. From the data available it was possible to estimate that 98 per cent of the pore area distributed among holes large enough for the diffusion of acetone was unavailable for the passage of glycerine and that only 0.3 per cent of the pore area available for acetone could be utilized by glucose. In trying to correlate the ratio between the diffusion rates of two different

substances with the characteristic concentration potential (*Co P*) given by the same membrane it was found (1) that with the acetone-glycerine ratio there is no correspondence (2) that with the acetone-glucose ratio a suggestive relation exists and (3) that with the glycerine-glucose ratio a definite correspondence can be shown, the higher ratios being obtained only with membranes giving high *CO P* values. A rational explanation for these facts was proposed.

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DARK ADAPTATION IN AGRIOLIMAX.

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I.

When the slug *Agriolimax*, previously dark adapted, is allowed to creep geotropically upward upon a vertical plate, lateral illumination forces it to pursue a path at an angle (β) to the perpendicular (*cf.* Wolf, 1926–27) and away from the light. The magnitude of this angle decreases with time (Wolf and Crozier, 1927–28), due to photic adaptation in the eye (Crozier and Wolf, 1927–28). The change with time during continuous exposure is adequately accounted for, quantitatively and very simply, by the assumption that the path at any moment is the resultant of 2 vectors, respectively those determined by geotropic and by phototropic excitation, and that these vectors express quantitatively the effects of the corresponding excitations (Wolf and Crozier, 1927–28). The geotropic excitation is taken to be constant, while the phototropic excitation decreases exponentially with time, due to the first order character of the light adaptation. These assumptions lead to an expression for β as a function of time,

$$\text{time} = a - \frac{1}{K} \log \tan \beta, \tag{1}$$

which is very satisfactorily obeyed; and to the expectation that the *rate* of adaptation, K in equation (1), should be practically a logarithmic function of the light intensity (over intermediate ranges), which is also substantiated. The assumptions underlying this treatment are that the intensity of excitation is proportional to $-\frac{ds}{dt}$, where S is the substance which is decomposed photochemically,

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and that with the animals fully dark adapted at the start $-\frac{ds}{dt}$ is *effectively* proportional to $[S]$ at each moment, any reverse reaction serving to regenerate S being negligible under the conditions (at lower temperatures, when dark adaptation is known to be quite slow). It was pointed out (Wolf and Crozier, 1927–28) that this type of experiment can be reversed for the study of dark adaptation. It is important to determine the characteristics of the receptor mechanisms concerned with continuous excitation by light, as in phototropism, and we have accordingly made experiments regarding the kinetics of dark adaptation in *Agriolimax*. The continuous nature of the photic excitation is proved by the character of the path of orientation. The orientation is fully accounted for by the assumption that the difference in tonus of the musculature which results in turning is proportional to the photic excitation. The results demonstrate one further respect in which the receptor machinery for phototropic excitation and that for differential sensitivity, as studied by Hecht (1918–19; 1926–27) in several forms, are fundamentally akin. They may also be invoked as supplying an additional chapter in the development of the prediction of conduct in compound fields of stimulation (Crozier, 1923–24; 1926–27; Crozier and Pincus, 1926–27; Crozier and Stier, 1927–28).

II.

Agriolimax was allowed to become light-adapted by exposure for at least 2 hours to bright daylight at a window facing an unclouded sky. It is necessary to make sure at intervals that the slugs are creeping, with eye-stalks everted. Exposure to direct sunlight is quickly injurious, but illuminations of about 1,000 m. c. proved non-toxic and adequate (*i.e.*, sufficiently high) for our purpose. Light-adapted individuals were then taken in succession and placed in darkness for 5 minutes each. A curve of light adaptation was then obtained for each, by measuring the divergence from perpendicular creeping upon a moist vertical plate of ground glass, as earlier described (Wolf and Crozier, 1927–28). The adapting light was of 29.4 f. c. The data from a sample of 10 individuals treated in this way show good agreement, and the readings of the angle of divergence (β) at successive minutes during light adaptation are accordingly averaged. The

TABLE I.

Amplitudes of response (β) at successive intervals in lateral light of 29.4 f.c. intensity, beginning at various levels of dark adaptation. (The magnitudes of β have been checked by further groups of experiments.)

Time in darkness	No. of individuals	Time in light	β
min.		*min.*	
5	16	0.5	31.98°±0.995
		1.5	21.23°±1.00
		2.5	13.07°±1.02
10	15	0.5	40.28°±1.208
		1.5	26.34°±0.605
		2.5	18.97°±0.477
		3.5	12.62°±0.639
15	17	0.5	44.06°±1.05
		1.5	31.87°±0.702
		2.5	25.0° ±0.838
		3.5	18.0° ±0.526
20	10	0.5	59.45°±1.25
		1.5	45.80°±1.28
		2.5	34.30°±1.20
		3.5	25.80°±1.02
25	8	0.5	58.0° ±1.25
		1.5	46.63°±1.14
		2.5	35.81°±0.940
		3.5	26.44°±1.05
30	10	0.5	59.25°±1.97
		1.5	42.60°±1.30
		2.5	30.25°±1.45
		3.5	20.90°±1.24
45	17	0.5	61.60°±1.05
		1.5	49.90°±1.17
		2.5	34.62°±0.723
		3.5	25.43°±0.821
60	14	0.5	62.50°±1.75
		1.5	47.2° ±0.936
		2.5	34.33°±1.03
		3.5	24.89°±0.529

tests are then repeated with similar slugs each dark-adapted for 10 minutes, and so on for succeeding intervals up to 60 minutes (Table I). The temperature in the dark room was held at 20.5° to 22.0° throughout. At this temperature the rate of increasing effectiveness of photic excitation is still very slow by comparison with that of adaptation by light.

It is impossible to carry through such series of observations, or rather to interpret the results in a simple and direct way, when single individuals are tested at different times during their dark adaptation (as subsequently discussed), and physical limitations make it necessary to deal with relatively small groups of individuals at a time (their respective intervals in darkness being properly spaced and controlled). The data for each curve of light adaptation has therefore been based, as a rule, upon a group of ten slugs. Individual differences, of quantitatively perceptible order but of consistent sense, expose the interpretation of such data to certain hazards. At each desired point on the final curve of dark adaptation, therefore, the tests were repeated with new groups of individuals. Aside from possible but slight variations in the intensity of the test light, and of the adapting illumination, the existence of diurnal variations in photic excitability must be guarded against; and, more important in a practical way, diurnal fluctuations in geotropic responsiveness. It is known, from our own experience with *Agriolimax* and other slugs, that the geotropic response is more powerfully in evidence, or—what may not be the same thing—more frequently expressed,—toward evening and after nightfall than in the early morning hours. Diurnal fluctuations of activity have long been noted in other forms. In our earlier work such changes have been discounted by running the experiments between hours 11 a.m. and 6 p.m., and by using a large number of series of observations spread more or less uniformly over this interval.

The derivation of equation (1) assumed that g, the geotropic vector, remained constant. It is also assumed that the threshold value of the photic excitation, required to produce any phototropic orientation on the vertical plate, is not a value to be subtracted from the total photic excitation, but that if effective at all the photic stimulation has its full proportionate value. Questions concerning the central nervous interplay between photic and gravitational stimulations receive some

further elucidation in experiments subsequently made with *Limax*, to be treated in another place. Variations in this threshold quantity would not affect the intercept on the t axis in Fig. 1, nor the slope of the curve of light adaptation.

We have been fully alive to the fact that in treating the photic excitation no account has been taken of the inclination of the receptive surface to the path of the light. One can suspect that change in this angle would alter the magnitude of our L. But the question is complicated by the fact that the eye-tentacles of *Agriolimax* diverge further as light adaptation increases, so that the angle between the eye-surfaces ("*H*" of a previous paper—Crozier, 1925–28) decreases as the slug comes to orient more nearly straight upward. This would tend to increase L illegitimately, and to cause curves such as those in Fig. 1 to be *slightly* concave upward. In a number of instances this appears to be true, and in no instance is the reverse true. But the effect, if real, is of small magnitude, with the orienting light of moderate intensity; and it may possibly be accounted for in other ways (*cf.* § VII).

III.

The notion that excitation by light is a photochemical matter, and that the maintenance of photic reactivity and its recovery after exhaustion may be in some fashion connected with a reversible photochemical receptor system seems to have been held in a very general form since Hering. Growing knowledge of the chemical effects of light made it attractive to translate such ideas into photochemical terms. An early attempt to treat the question precisely and specifically, but with some important deficiencies, was made by Müller (1896). To explain the simultaneous exhibition of capacity to react to a sharp decrease in light intensity and also to be excited to phototropic movements by the continuous action of light, as seen in the integument of certain holothurians, it was suggested that the same photochemical system, if part of a reversible reaction of a certain kind, might be conceived to serve for both modes of stimulation (Crozier, 1915). Hecht[1] has been able to achieve the step-by-step proof, in a very satisfying and convincing manner, that a particular

[1] Hecht, 1918–19; 1919–20, *a*, *b*; 1922–23; 1925–28; 1926–27.

type of reversible photochemical system underlies photic excitation of *Mya*, *Ciona*, *Pholas*, and the human eye. The properties of this system permit the prediction and the rational quantitative interpretation of new and unexpected characteristics of the stimulation process. A major property of the underlying mechanism is adequately expressed (Hecht, 1918–19; 1922–23) in the following paradigm, where S represents primary photosensitive substance, P and A two of the products of its photolysis:

$$S \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} P + A \qquad (2)$$

The system is reversible, and with time, under continuous constant illumination, there is established a stationary state ("equilibrium"). The "dark" process, of which the kinetics serves to describe the course of dark adaptation, is definitely bimolecular (Hecht, 1926–27), with high temperature characteristic; the "light" reaction is of first order and negligibly influenced by temperature (Hecht, 1919–20, *a*).

These relationships have been established most elaborately by means of experiments with organisms in which the speed of response following the delivery of a measurable quantity of light gives data necessary for the quantitative treatment, and in which light adaptation is rapid and photic excitation therefore necessarily discontinuous. To carry over such considerations to the analysis of continuous excitation, as in phototropic stimulation, it has been necessary to find phototropic organisms in which the rate of light adaptation is measurable, neither inconveniently rapid nor too slow. The photic adaptation of *Agriolimax* has been followed by causing the phototropic influence of the light to work against the "brake" provided by a vectorially constant excitation of a different kind, namely that due to gravity, acting at right angles to the phototropic vector. The function of such a "brake" in contributing to the significance of the measured orientations has been discussed previously in relation to circus movements (Crozier and Federighi, 1924–25). Data necessary for treatment of the light adaptation are gotten from measurements of the resultant angles of orientation as related to time and to intensity (Wolf and Crozier, 1927–28). Such measurements are possible because the rate of light adaptation, especially at temperatures in the neighborhood of 15° or slightly above, is so very much faster than

that of dark adaptation, as adequate tests showed. It was easily established that the rate of light adaptation is very little influenced, if at all perceptibly, by temperature; and that its course follows that of a first order reaction in which the "velocity constant" is a linear function of the logarithms of the light intensity. These phenomena therefore parallel in an exact manner the properties of the "light" reaction already discussed.

The interpretation of dark adaptation requires measurements of the photic excitability at successive intervals following exclusion of light from individuals previously light-adapted. To reduce the probability of adventitious errors of estimation the experiments were made at a temperature high enough to bring the time for practically complete dark adaptation down to a little over 1 hour. At about 12°, the time required may be as long as 3 to 4 hours; at 20.5°–22°, the temperature prevailing throughout the present experiments, the time is less than half of this. Since light adaptation, *as measured by the power to influence geotropic creeping on a vertical surface*, is effectively complete within about 5 minutes, as a rule, with high intensities, the experiments must, for simplicity, take the form of establishing curves of light adaptation as affected by known preceding periods in darkness. From such data it is required to deduce the ideal photic excitability at the last moment in darkness, before the exposure has begun, or in other words the kinetics of the "dark" reaction. The necessity for speedy and precise manipulation has made it undesirable, for the present, to vary the light intensity from moment to moment in such a way as to keep the angle β constant. This alternative method has certain theoretical advantages, however, and subsequently will be used.

IV.

Two aspects of the measurements at once present themselves—the *rates* of light adaptation after various periods in the dark, and the magnitudes of β at the first instant of exposure to the light. It is apparent from Fig. 1 that the course of light adaptation after given conditions of adjustment in the dark adheres to the equation previously obtained,

$$\text{time} = a - \frac{1}{K} \log_{10} \tan \beta, \qquad (2)$$

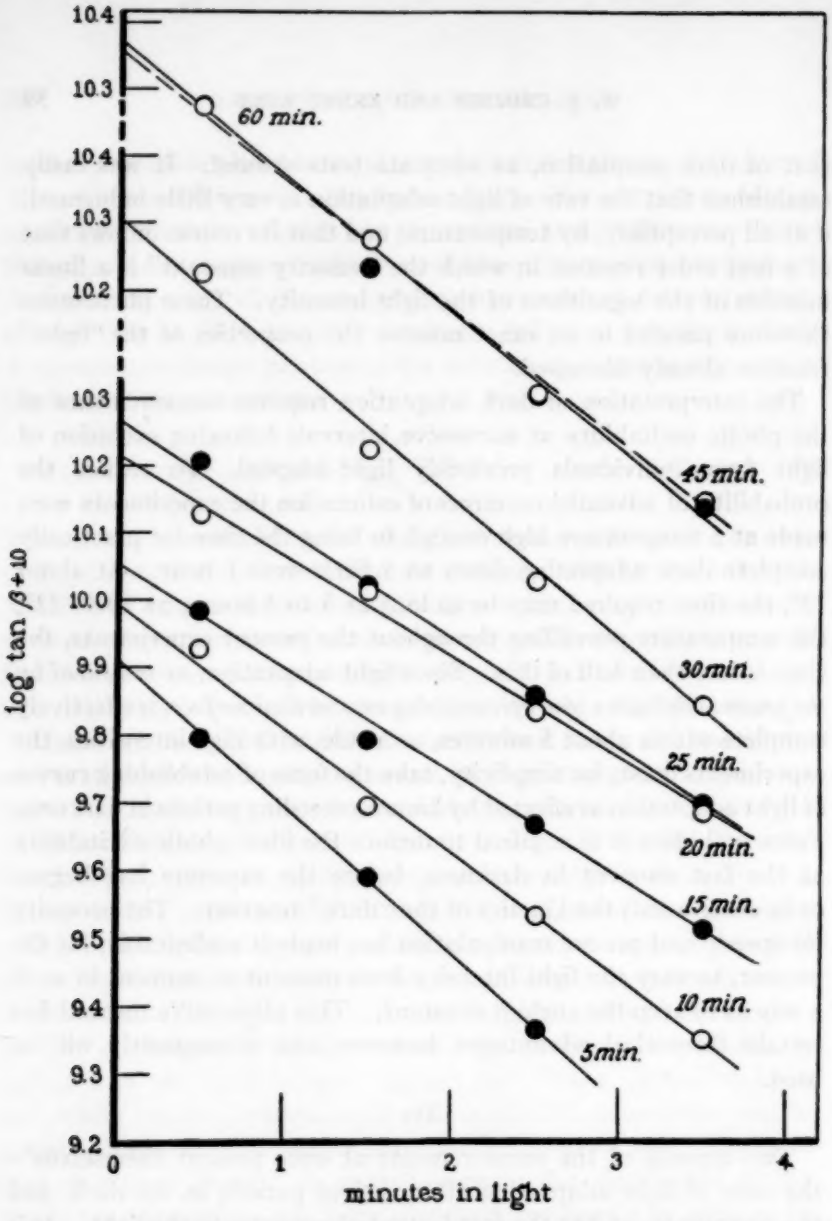

FIG. 1. The angle (β) of orientation of *Agriolimax* away from the vertical path on a perpendicular plate, as enforced by exposure to horizontal light of 29.4 f.c. on the right side, at successive minutes during the exposure, following increasing residence in darkness (5 to 60 minutes) of slugs initially light-adapted. Time of exposure plotted against log tan β gives graphs sensibly rectilinear. The intercepts of these graphs on the ordinate axis give a means of estimating the photic excitability at the very first moment of exposure to light (see text). (The ordinate axis has been shifted vertically, for "30 min." and for "45 min." and "60 min.," to obviate overlapping.)

and that K, the rate coefficient of light adaptation, and a, the intercept on the β axis corresponding to zero time of exposure to light, each vary with the extent of dark adaptation. From the assumptions made and tested in the previous papers, we may write

$$\tan \beta = \frac{L}{g}, \tag{3}$$

where L and g represent respectively the photic and the gravitational orientation vectors. If g is constant, $L = g \tan \beta$. L is taken to be a measure of the average amount of photosensory substance present,

TABLE II.

Intercepts on the $\log_{10} \tan \beta$ axis (i°, $\log_{10} \tan \beta_i$) at $t = 0$ exposure to light (29.4 f.c.), following increasing residence (t) in darkness of initially light-adapted slugs. $R = \frac{KL_\infty}{KL_t}$; KL_∞ = intercept for completely dark-adapted animals.

t	$\log_{10} \tan \beta_i$	β_i	R
min.			
5	9.910–10	39.1°	3.020
10	9.996	44.7°	2.477
15	10.059	48.9°	2.143
20	10.216	58.7°	1.528
25	10.264	61.5°	1.337
30	10.305	63.7°	1.216
45	10.350	65.9°	1.096
60	10.362	66.5°	1.067
∞	10.390	67.9°	1.000

S, or more precisely as the average value of $-\frac{ds}{dt}$, during the interval over which β is estimated. This is the amount of substance the increase of which, in terms of the conception of a reversible photochemical system, we desire to follow during dark adaptation. The two asumptions as to the nature of L will be tested separately.

Taking the intercepts on the $\log_{10} \tan \beta$ axis of Fig. 1, and from these obtaining $\tan \beta$, we have numbers proportional to the concentrations of S present at the beginning of the test-exposures, if the "dark" reaction is relatively negligible; this follows from the primary assump-

tion (Wolf and Crozier, 1927–28) that the phototropic excitation in any short interval is proportional to $-\frac{ds}{dt}$, and thus to the amount of S photolysed in unit time. Strictly speaking, this of course may not be entirely accurate, since the angle β is estimated over a finite length of time and of path; but deficiencies arising in this way are lost among the individual variations of response and are of no moment

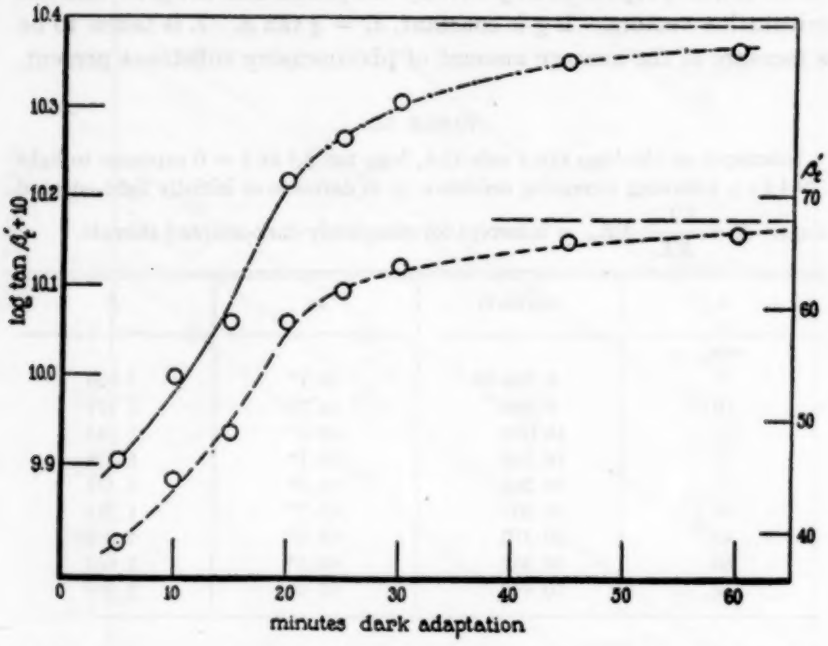

FIG. 2. Curves showing the increase of the intercept angle (β_t'), and of log tan β_t', as a function of time in the dark subsequent to prolonged exposure to light.

for our present purpose. The values of tan β corresponding to $t = 0$ increase regularly with time of residence in darkness (Table II and Fig. 2), and the adaptation is therefore an orderly process. The type of reaction which describes the kinetics of dark adaptation, and thus of the "dark" reaction in the absence of light, may be ascertained from the data in Table I and Fig. 2. Since tan β is proportional to L, the intensity of excitation, and this by assumption to $-\frac{ds}{dt}$ and

so to S_t, the amount of photosensitive substance present at the moment, we might write

$$\frac{\tan \beta_\infty}{\tan \beta_t} = \frac{L_\infty}{L_t} = R, \tag{4}$$

where β_t is the angle given by the intercept on the log tan β axis in Fig. 1, —*i.e.*, corresponding to zero time in the light, after t minutes dark adaptation, and β_∞ is the intercept angle after complete dark adaptation. Thus we have a measure of the photic sensitivity at the first moment of exposure to the test light. From (4), since $L_t \propto S_t$, S_t being the concentration of S after time t in the dark,

$$\frac{[S_\infty]}{[S_t]} = R; \tag{5}$$

tan β_∞ is obtained from observations on animals fully dark-adapted (Wolf and Crozier, 1927–28).

If the reformation of S in the dark were a first order process, log S_t should increase linearly with time. We do not know S_t in absolute units, but we have a proportionate measure of it from (4) and (5). More precisely, if we assume $R = \dfrac{dx'/dt}{dx_t/dt}$, where dx'/dt = the rate of photolysis of S when dark adaptation is at first *complete*, and therefore $\propto [S_\infty]$, then $-\dfrac{dS_t}{dt} = \dfrac{dx_t}{dt} = \dfrac{kS_\infty}{R}$; this, on the assumption of the moment, would be equal to $K_2 (S_\infty - S_t)$, and log $\dfrac{S_\infty}{R}$, or log R, should still be a linear function of time in the dark. The assumption of a "monomolecular" formation of S can therefore be tested by plotting log $\dfrac{S_\infty}{R}$, or log R, against time of dark adaptation. As Fig. 3 shows, the relationship is certainly not rectilinear, and this assumption may be dismissed; moreover, it would leave unexplained the changes in *slope* of the curves of light adaptation.

If the formation of S in the dark be assumed bimolecular, corresponding to

$$S \leftarrow P + A,$$

then $(S_\infty - S_t)$ must decrease hyperbolically with time in the dark,

or the graph of $1/(S_\infty - S_t)$ *vs.* t must be linear; but $S_\infty - S_t = (R-1)(S_t) = \frac{(R-1)(S_\infty)}{R}$, and therefore $\frac{1}{S_\infty - S_t} = \frac{1}{S_\infty} \cdot \frac{R}{(R-1)}$; since $\frac{1}{S_\infty}$ is by assumption constant, the plot of $\frac{R}{(R-1)}$ against time in

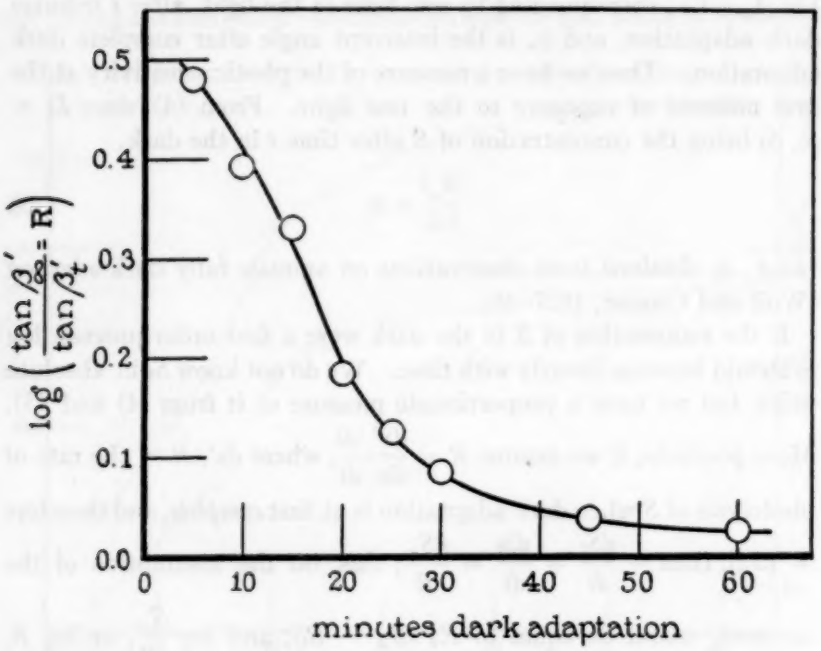

FIG. 3. If the course of dark adaptation were describable as a first order process log R should be linearly proportional to time in the dark,—which is obviously not the fact. R is the ratio between initial excitability after complete dark adaptation and that after t minutes in darkness. The excitability may be taken as proportional to $[S]$, the concentration of photosensitive substance at the moment, or to $-\frac{ds}{dt}$.

darkness should show a linear increase. As is evident in Fig. 4, the relationship is anything but linear; in fact the curve shows a distinct sigmoid flexure and apparent relative inhibition of formation of S at the start. If we prefer to regard L as proportional to $-\frac{ds}{dt}$, then it is

easily shown that R, for a second order process, should be linearly proportional to t—which is certainly not the fact.

To account for the form of this curve it is necessary, as the simplest assumption, to invoke some type of autocatalysis. If the "dark" reaction were first order and autocatalyzed by S, then

$$-\frac{\delta x}{\delta t} = K_2 (S_\infty - x)(x) + K'x \tag{6}$$

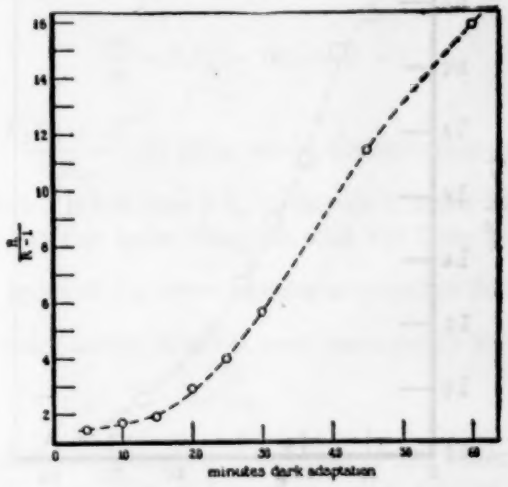

FIG. 4. Were the course of dark adaptation susceptible to formulation as a second order reaction, then $\frac{R}{R-1}$ should be a rectlinear function of confinement to darkness. Clearly, such formulation fails.

where x = concentration of A and of P; during exposure to light, also

$$\frac{\delta x}{\delta t} = K_1 (S_\infty - x), \text{ and}$$

$$\frac{dx}{dt} = K_2 (S_\infty - x)(x) + K'x - K_1 (S_\infty - x) = (K_2 x - K_1)(S_\infty - x) + K'x \tag{7}$$

If for simplicity we assume, as has usually been done in the treatment of first order autocatalysis by a reaction product (*cf.* Crozier, 1926–27).

that K' is negligibly small, then the course of dark adaptation (K_1 being then $\equiv 0$) is given by

$$\frac{\delta x}{\delta t} = K_2 (S_\infty - x) (x); \tag{8}$$

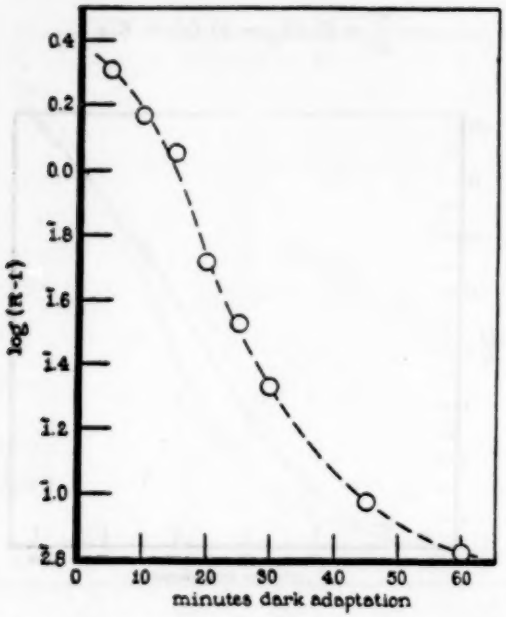

FIG. 5. The "dark" formation of photosensitive substance cannot be accounted for as the result of a first order process with positive autocatalysis, since log $\frac{x}{S_\infty - x}$ (or its proportionate equivalent, log $(R - 1)$), is not a linear function of time in the dark.

and, as is well known, the graph of log $\frac{x}{S_\infty - x}$ against time should show a linear decrease (if the value of S_∞ is properly chosen); in terms of our present notation, from (4), log $\frac{x}{S_\infty - x} \equiv$ log $(R - 1)$. It is clear from Fig. 5 that the graph is not rectilinear; nor is its approach to rectilinearity at all improved by the assumption of other

(slightly different) magnitudes for S_∞. Moreover, even assuming for a proposed autocatalytic effect of first order type the more complete differential equation

$$\frac{\delta x}{\delta t} = K_1 (S_\infty - x)(x) + K'x,$$

the inflection point in the sigmoid curve relating S to t during dark adaptation must come at

$$\frac{d^2s}{dt^2} = K_2 S_\infty - 2K_2 x + K' = 0,$$

whence $x = \dfrac{K_2 S_\infty + K'}{2K_2}$; in other words, the inflection must come at a point such that S is less than $\frac{1}{2} S_\infty$ (although it might be very close to $S = \frac{1}{2} S_\infty$); but this quite disagrees with the facts (*cf.* Fig. 4, and Fig. 6). In terms of the more rigorous assumption that $L \propto \dfrac{ds}{dt}$, the idea of first order autocatalysis is even more poorly substantiated.

V.

We must therefore give up a conception of first order formation of S during dark adaptation, and turn to a second order process (with auto-acceleration by S) as possibly giving the simplest description of the facts. The integral equations for such processes are not easy to handle, and indeed must be devised for the occasion; but they provide several decisive tests which are of considerable interest. These tests are of a nature which tends to remove one's suspicion that the agreement between formula and experiment might be merely a descriptively successful accident.

We will assume that the differential expression for the second order autocatalysis may be written

$$\frac{ds}{dt} = K_2(S)(x^2) = K_2 (S_\infty - x)(x^2),$$

neglecting, as before, the additional catalytic effect more properly

cared for by the introduction of another velocity constant. Then for the rate of change of [S] in the light we will have

$$-\frac{\delta s}{\delta t} = K_1 (S_\infty - x),$$

and for the dark reaction

$$\frac{\delta s}{\delta t} = K_2 (S_\infty - x) (x^2),$$

and therefore

$$\frac{ds}{dt} = K_2 (S_\infty - x) (x^2) - K_1 (S_\infty - x), \tag{9}$$

or

$$\frac{dx}{dt} = -\frac{ds}{dt} = K_1 S_\infty - K_1 x - K_2 S_\infty x^2 + K_2 x^3.$$

When x is very small (relatively complete dark adaptation) $-\frac{ds}{dt}$ on exposure to light will thus depend largely on $K_1 (S_\infty - x)$, and (other things equal) will be large (*cf.*, Wolf and Crozier, 1927–28); when x is large $-\frac{ds}{dt}$ will be low and its rate of change in light will be slowed, roughly in proportion to x^2. This corresponds in a crude way to the increase in the slope of the curve of light adaptation as dark adaptation proceeds (Fig. 1).

The curve describing the change of photic excitability with increasing dark adaptation is given, on this assumption, by

$$y = -\frac{ds}{dt} = K_1 S_i - K_2(S_i) (S_\infty - S_i)^2, \tag{10}$$

$$= K_1 S_i - K_2 S_\infty^2 S_i + 2 K_2 S_\infty S_i^2 - K_2 S_i^3,$$

where y, $= -\frac{ds}{dt}$, is proportional to L at the first instant of exposure to the light, and so, as before, to $1/R$. As [S] increases, $\frac{dy}{ds}$ is the slope of $1/R$ plotted against S as S increases during the time of dark adapta-

tion. The slope of $1/R$ passes through an inflection point, since dy/ds is maximum at

$$d^2y/ds^2 = 0 = -4K_2S_\infty + 6K_2S, \text{ or } S = \tfrac{2}{3}S_\infty,$$

whence

$$ds/dt = \tfrac{2}{3}\frac{ds_\infty}{dt}, \; ds/dt \Big/ ds_\infty/dt = 1/R = \tfrac{2}{3}.$$

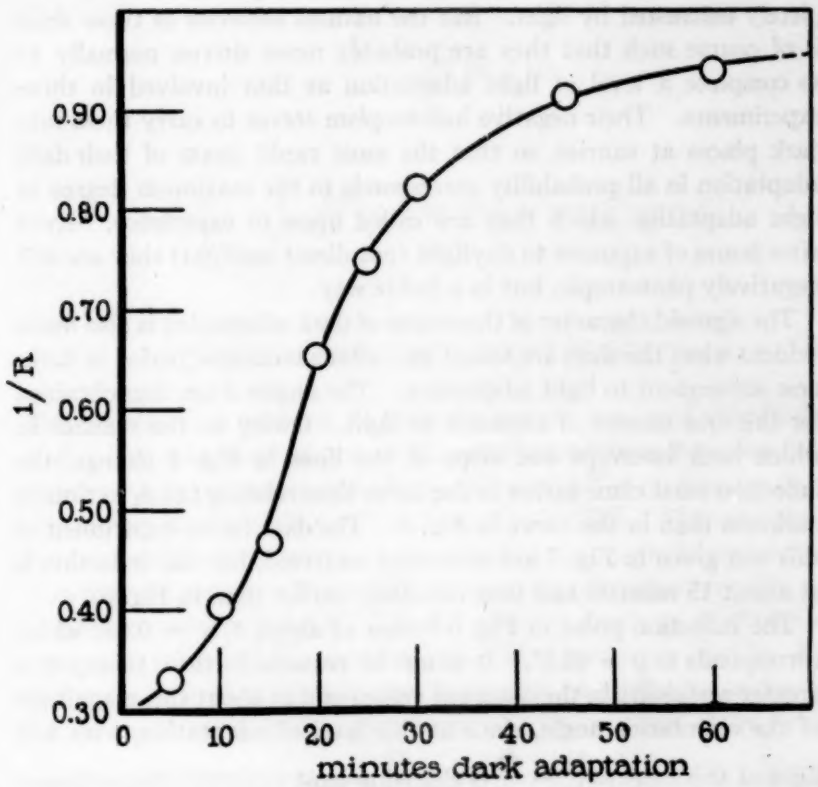

FIG. 6. The "dark" restitution of S through a second order reaction with positive autocatalysis would require that the photic excitability, $-\frac{ds}{dt}$, or its proportionate equivalent $1/R$, should exhibit a maximum velocity of change when $[S_i]$ has been brought to a little less than $\frac{2}{3}$ $[S_\infty]$; $1/R$ should therefore pass through an inflection point when $1/R$ = a little less than $0.67-$; the inflection is found at $1/R = 0.64$.

If the suppressed velocity constant K' be taken into account, x at the inflection point $= \frac{1}{3}\left(\frac{K'}{K_2} + S_\infty\right)$ and $\frac{1}{R}$ must be a little *less* than $\frac{2}{3}$. This graph is given in Fig. 6. The general form of the curve is as expected, and the empirically determined inflection point is at about 0.63 or slightly more. The agreement is therefore strikingly good.

It might be said that the most sensible thing would be for the animal to be *most rapidly* dark-adapting when its receptors are most completely exhausted by light. But the natural behavior of these slugs is of course such that they are probably never driven normally to so complete a level of light adaptation as that involved in these experiments. Their negative heliotropism serves to carry them into dark places at sunrise, so that the most rapid phase of their dark adaptation in all probability corresponds to the maximum degree of light adaptation which they are called upon to experience. Even after hours of exposure to daylight (not direct sunlight) they are still negatively phototropic, but in a feeble way.

The sigmoid character of the course of dark adaptation is also made evident when the slugs are tested *once* after a measured period in darkness subsequent to light adaptation. The angles β are thus obtained for the first minute of exposure to light. Owing to the manner in which both intercept and slope of the lines in Fig. 1 change, the inflection must come earlier in the curve then relating $\tan \beta_1$ to time in darkness than in the curve in Fig. 6. The data for an experiment of this sort given in Fig. 7 are somewhat scattered, but the inflection is at about 15 minutes and thus definitely earlier than in Fig. 6.

The inflection point in Fig. 6 comes at about $1/R = 0.63$, which corresponds to $\beta = 44.5°$. It would be reasonable then, to expect a greater *variability* in the observed values of β at about this magnitude of the orientation angle, since at this level of adaptation, with test light of this intensity, $-\frac{ds}{dt}$ is changing most rapidly. Inspection of the probable errors (Table I) shows that the P. E. declines as β becomes less, but there are few measurements above $\beta = 45°$; in earlier series (Wolf and Crozier, 1927–28) the effect predicted is reasonably clear.

VI.

The integral for the curve of dark adaptation is obtainable only in an inconvenient form. Assuming that $R = \frac{S_\infty}{S_t}$

$$\frac{ds}{dt} = K_2 S_t (S_\infty - S_t)^2 = \frac{K_2 S_\infty^2}{R^2} (R-1)^2 = -S_\infty R^{-2} \frac{dR}{dt},$$

therefore

$$-\frac{dR}{dt} = \frac{K_2 S_\infty^2}{R} (R-1)^2$$

and

$$-K_2 S_\infty^2 t = \int \frac{R dR}{(R-1)^2} = \log (R-1) - \frac{1}{(R-1)} + C,$$

or

$$K_2 S_\infty^2 (t_1 - t_2) = \frac{R_2 - R_1}{(R_1 - 1)(R_2 - 1)} + \log \frac{(R_1 - 1)}{(R_2 - 1)} \qquad (11)$$

This enables us to evaluate K_2 from the directly measured magnitudes of R. The calculated values of K_2, as obtained from equation (11) and the tabulated values of R (Table II), taking $t_1 = 60$ minutes dark adaptation, show satisfactory constancy (Table III). This reference-point is not the most accurately established, but permits computing from the most points. Values of R read from the smooth curve (Fig. 6) give better constancy for K''. Slight errors in obtaining R affect the computed value of K'' considerably. The systematic rise in K'' during the first 15 minutes in darkness might be explicable as due to the failure to consider the suppressed velocity constant in the auto-catalytic formula (this makes $\frac{ds}{dt}$, hence $\frac{1}{R}$, appear "large"). Moreover, and this is most important, the derivation has assumed that the "dark" reaction is insignificant even at low concentrations of S, which is inadequate, and results in K'' passing through a maximum.

The complete expression for the rate of light adaptation is even more unmanageable. But several of its properties may be deduced, which

is all that is necessary for our purpose. If the conceptions underlying the analysis are correct, we must expect the rate of light adaptation to be a minimum when the rate of "dark" formation of S is highest. The change in the slopes of the curves of light adaptation (Fig. 1)

TABLE III.

Values of the velocity constant for dark adaptation, from $K_2S_\infty^2\ (t_1 - t_2) = \frac{R_2 - R_1}{(R_1 - 1)\ (R_2 - 1)} + \log \frac{(R_1 - 1)}{(R_2 - 1)}$, using ascertained values of R [Table II], taking $t_1 = 60$ minutes, and assuming $R = S_\infty/S_t$ (see text).

$t_1 - t_2$	$K_2S^2_\infty = K''$
min.	
15	0.320
30	0.341
35	0.358
40	0.314
45	0.294
50	0.267
55	0.241

should therefore supply an independent check on the interpretation. From equation (6),

$$-\frac{ds}{dt} = K_1S_\infty - K_1x - K_2S_\infty x^2 + K_2x^3;$$

this should give a clue to singularities in the curve relating $-\frac{ds}{dt}$ to changes in the concentration of x, for which the slope is

$$-\frac{d^2s}{dtdx} = -K_1 - 2K_2S_\infty x + 3K_2x^2 \qquad (12)$$

For a maximum or minimum the next differential must be equatable to zero, and for a minimum $-\frac{d^3s}{dtdx^2}$ must be positive.

$$-\frac{d^3s}{dtdx^2} = -2K_2S_\infty + 6K_2x,$$

therefore

$$x = \frac{1}{3} S_\infty,$$

which corresponds to a minimum since

$$-\frac{d^4s}{dtdx^3} = 6K_3.$$

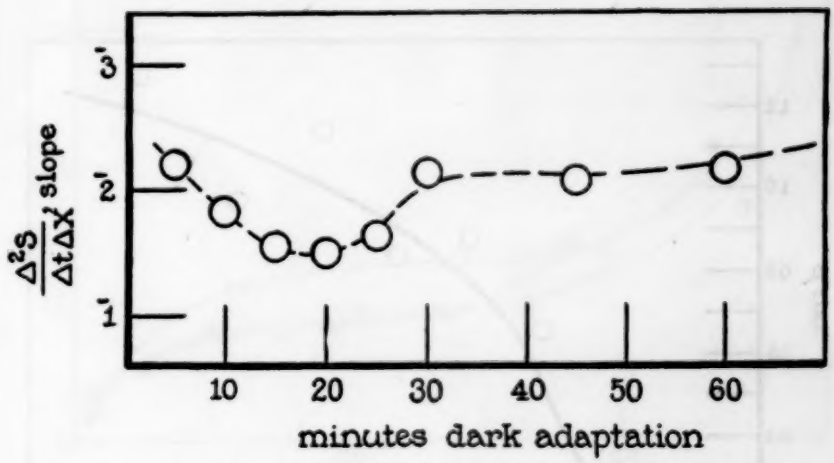

FIG. 7. The second order "dark" formation of S requires that the rate of light adaptation $\left(i.e., \text{change of } -\frac{ds}{dt}\right)$ should be a *minimum* when $\frac{ds}{dt}$ is increasing most rapidly. The minimum found shows precise agreement with this requirement.

The fuller formulation is

$$-\frac{ds}{dt} = K_1 (S_\infty - x) - [K_2 + K_3(S_\infty - x)] x^2$$

$$= K_1 S_\infty - K_1 x - (K_2 + K_3 S_\infty) x^2 + K_3 x^3;$$

$$\frac{d^2s}{dtdx} = -K_1 - 2 (K_2 + K_3 S_\infty) x + 3K_3 x^2,$$

$$\frac{d^3s}{dtdx^2} = -2 (K_2 + K_3 S_\infty) + 6K_3 x;$$

at inflection,

$$6K_3x = 2(K_2 + K_3S_\infty)$$

$$x = \frac{1}{3}\left(\frac{K_2 + K_3S_\infty}{K_3}\right) = \frac{1}{3}\left(\frac{K_2}{K_3} + S_\infty\right),$$

therefore

$$x = \text{a little} > \frac{1}{3}S_\infty.$$

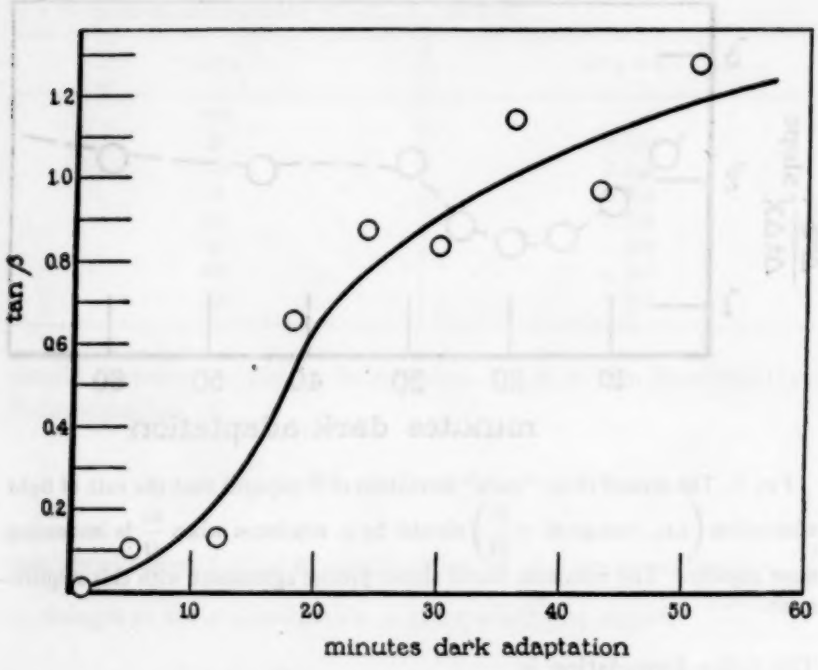

FIG. 8. A curve of dark adaptation as derived from single measurements of β with a number of slugs during the first minute of exposure to light subsequent to various periods in darkness following practically complete light adaptation. In this case, β corresponds to the values at 0.5 minute in Table I. Theory requires that the inflection of this graph should occur relatively earlier than in Fig. 2, which is the fact.

Hence the *rate of light adaptation*, with the test light of constant intensity, must pass through a minimum when x (*i.e.*, $S_\infty - S_t$,

or $S_\infty \frac{(R-1)}{R}$, has been reduced to slightly greater than $\frac{1}{2} S_\infty$. The minimum found (Fig. 7) is at $t = 20$ minutes or a little less; this corresponds precisely to the region of the inflection point in the previous curve (Fig. 6) describing formation of S in the dark, and indicates that the *rate* of light adaptation is least when the calculated

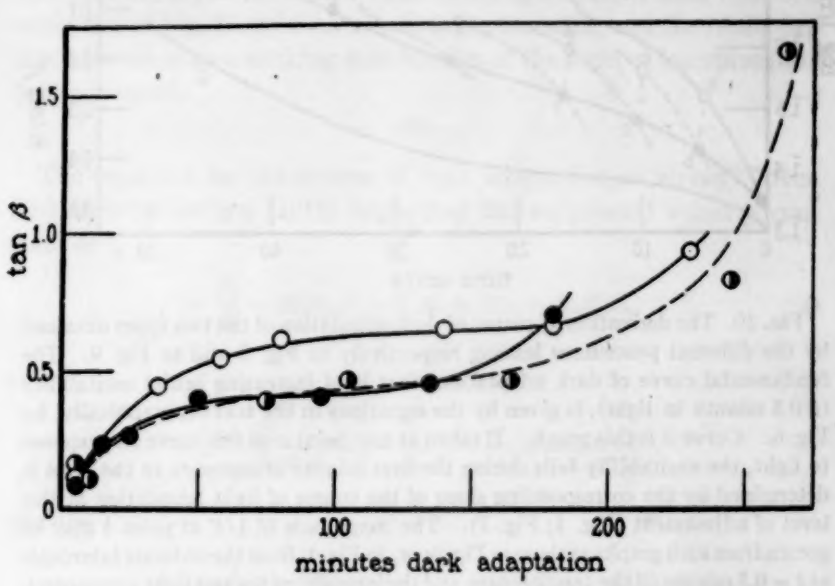

FIG. 9. Curves of dark adaptation as derived from repeated tests with the same individuals, at intervals during their photic adjustment in the dark. The shape of these graphs is to be contrasted with that in Fig. 8. The explanation is given in Fig. 10. (These curves were obtained from experiments at a temperature of about 15°, hence the time axis is extended by comparison with those earlier discussed.)

rate of formation of sensitive material is increasing most rapidly. The result is therefore quite reasonable.

Even if the tests for dark adaptation are made by the method of successive exposures of the same individuals to light during the progress of its dark adaptation, it is to be expected from the foregoing results that the change of the angle β should give a sigmoid curve when plotted against time. But since a certain amount of light

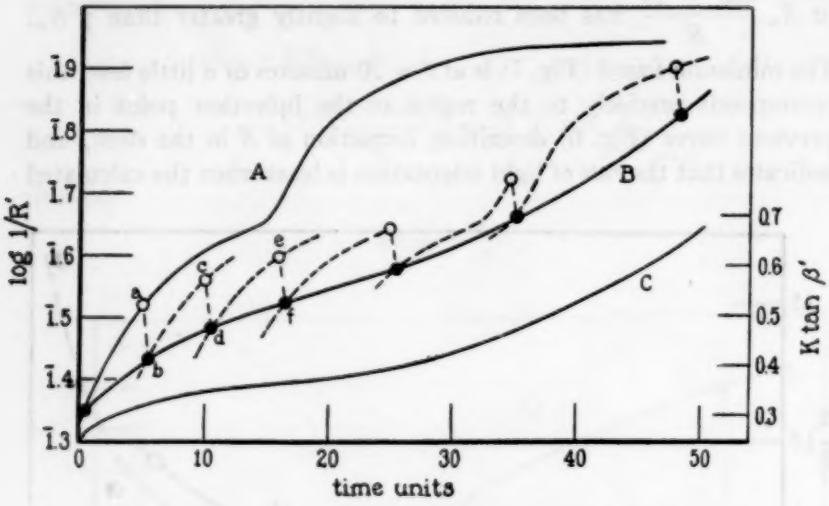

FIG. 10. The derivation of curves of dark adaptation of the two types obtained by the different procedures leading respectively to Fig. 8 and to Fig. 9. The fundamental curve of dark adaptation, that is of increasing initial excitability (β 0.5 minute in light), is given by the equations in the text or, graphically, by Fig. 6. Curve *A* is this graph. If taken at any point *a* on this curve and exposed to light, the excitability falls during the first minute of exposure to the level *b*, determined by the corresponding slope of the course of light adaptation at this level of adjustment (Fig. 1; Fig. 7). The magnitude of $1/R$ at point *b* may be gotten from such graphs as those in Fig. 8; or, in Fig. 1, from the ordinate intercepts at $t = 0.5$ minute (if the temperature and the intensity of the test light correspond; here, we are dealing only with the general *shape* of the resulting graphs). The curve of $1/R'$ is computed precisely in the manner of that for $1/R$, but on the basis of the ordinate intercepts (Fig. 1) at 0.5 minute exposure. On return to darkness, $1/R'$ begins again to increase, but now starts from level *b*. At intervals, this is all repeated. The continuous history deduced for $1/R'$ is then given by the *dotted line*. The observed change of excitability with time since first being placed in darkness should have the form given by the *heavy line*, which agrees with that found experimentally (Fig. 9); in the latter case the time axis is extended because the temperature was lower. The exact shape of curve *B*, particularly at the two ends, will depend on the frequency with which tests are made. There is thus a considerable interval over which the excitability does not change very much, when repetitive stimulation is employed. Curve *C* is curve *B* transformed to the same coordinates as Fig. 9.

adaptation is undergone during each interval of exposure, nothing can be gotten directly from such a curve (Fig. 9) whereby to test the quantitative formulation of the process. But it can be foretold that the shape of the curve under these conditions ought to be significantly different from that given in Fig. 7. Several curves obtained by taking single readings of β with slugs dark-adapted for different intervals are given in Fig. 8. The direct synthesis of such curves is explained in Fig. 10, without resort to the formulæ, and the result can fairly be taken as a striking justification of the form of interpretation here advanced.

VII.

The equation for the course of light adaptation as already given (10) may be written (again neglecting the suppressed velocity constant K'),

$$-\frac{ds}{dt} = 2K_2S_\infty S_t^2 - K_2S_t^3 + (K_1 - K_2S_\infty^2)S_t, \tag{12}$$

or

$$-t = \int \frac{ds}{2K_2S_\infty S_t^2 - K_2S_t^3 + (K_1 - K_2S_\infty^2)S_t},$$

whence

$$-t = \frac{1}{K_1 - K_2S_\infty^2}\left\{\frac{1}{2}\log\frac{S_t^2}{(K_1 - K_2S_\infty^2) + 2K_2S_\infty S_t - K_2S_t^2} + \frac{K_2S_\infty}{\sqrt{K_1K_2}}\tanh^{-1}\frac{K_2(S_\infty - S_t)}{\sqrt{K_1K_2}}\right\} + C,$$

where C is the constant of integration. In this case, $S_t = S_\infty$ when $t = 0$;

therefore

$$-t = \frac{1}{K_1 - K_2S_\infty^2}\left\{\frac{1}{2}\log\frac{K_1S_t^2}{S_\infty^2(K_1 - K_2S_\infty^2 + 2K_2S_\infty S_t - 2KS^2)} + \frac{K_2S_\infty}{\sqrt{K_1K_2}}\tanh^{-1}\frac{K_2(S_\infty - S_t)}{\sqrt{K_1K_2}}\right\} \tag{13}$$

At early stages of light adaptation, such as are measured in the curves in Fig. 1, beginning at any level of dark adaptation ($S_\infty - S_t$) does not change greatly; and if K_2 is quite small, as it is nown to be relative to K_1, then

$$-t = K''' \log S + K''', \text{ very nearly.}$$

It will be noticed that this is the relationship already illustrated in Fig. 1, although the *linearity* of the curve relating log tan β ($\equiv$, practically,[2] to log S) to time did not enter into the nature of the data used in the subsequent deductions.[3] Hence the conclusion now obtained from (11) is not to be regarded merely as "getting out something originally put into the formula."

VIII.

The present analysis of phototropic effects as influenced by adaptation is capable of being developed in several directions. We may mention, in addition to temperature relations, such points as: photic adaptation as affected by wave-length, and (possibly) polarization; dark adaptation subsequent to photic adaptation by light of particular wave-lengths as followed by means of recovery curves for excitation by light of other wave-lengths; and the changes of orientation when the geotropic vector is also varied. Experiments are being undertaken to explore these possibilities.

IX.

SUMMARY.

A method is described which measures the excitation of *Agriolimax* by light, during the progress of light adaptation, by assuming that the orientating effect of continuous excitation is expressed as a directly

[2] As $\log\left(-\frac{ds}{dt}\right) = \log [2K_2 S_\infty S_t^2 - K_2 S_t^3 + (K_1 - K_2 S_\infty^2) S_t]$; if K_2 is very small, this is nearly equivalent to $\log K_1 S_t$ for the same reason that the assumption of $R = \frac{S_\infty}{S_t}$ is very nearly sufficient (*cf.* §V).

[3] Because the same *kind* of result is obtained if the analysis be based merely upon the values of β during the first minute of exposure to light.

proportionate tension difference in the orienting muscles of the two sides of the body. The tendency toward establishment of such a tension difference is caused to work against a similar geotropic effect at right angles to the phototropic one. This enables one to study the kinetics of light adaptation, and of dark adaptation as well. The situation in the receptors is adequately described by the paradigm

$$S \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} P + A,$$

similar to that derived by Hecht for the differential sensitivity of various forms, but with the difference that the "dark" reaction is not only "bimolecular" but also autocatalysed by the reaction product *S*. The progress of dark adaptation is reflected (1) in the recovery of the *amplitude* of the orientation and (2) in the *rates* of light adaptation at different levels of the recovery; each independently supports these assumptions, for which the necessary equations have been provided. These equations also account for the relative variabilities of the angles of orientation, and, more significantly, for the two quite different kinds of curves of dark adaptation which are obtained in slightly different types of tests.

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GEOTROPIC ORIENTATION IN ARTHROPODS.

III. The Fiddler Crab Uca.

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I.

Relations between extent of upward geotropic orientation on an inclined plane and the intensity of the effective gravitational pull have been studied and formulated in several different cases.[1] The formulations depend upon the view that the limitation of orientation is determined by the distribution of the pull of the animal's weight upon the muscles (of the body or of the appendages) of the two sides during progression. One test of this conception is obtained by examining the forms of the several equations proposed as descriptive for the mechanically different instances in which the morphology of the organisms, or their methods of progression, require the deduction of diverse analytical expressions. In general, it cannot be supposed that for animals differing fundamentally in this respect the same empirical rules will be found connecting the inclination of the surface and the extent of orientation. Since it is often desired to employ convenient empirical rules for purposes to which more rational equations do not easily lend themselves, it becomes to a certain extent important, as well as interesting, to supply in this way a qualitative check upon the "reality" of the differences between empirical rules for orientation as found with different organisms.

The orientation of such brachyurans as the fiddler crabs is particularly significant in this connection, since they creep sideways, and with the body not in contact with the creeping surface. The lateral

[1] For citations of earlier papers *cf.* Crozier (1928); Crozier and Pincus, (1926–27, *a*; 1927–28); Wolf (1926–27); Crozier and Stier (1927–28; 1928–29).

mode of progression in relation to heliotropic movements is already known (*cf.* Holmes, 1908; Loeb, 1912). Questions arising here, as to the inner, nervous mechanisms of orientation may perhaps be approached by the method of compounding geotropic and phototropic effects (*cf.* Crozier and Pincus, 1926–27, *b;* Crozier and Stier, 1928–29; Wolf and Crozier, 1927–28), particularly since it may be possible by certain treatments to force creeping to occur in a cephalad direction and thus at right angles to the direction of the more usual method. Nor should it be overlooked that with such crustaceans it may be possible to make experiments designed to test quantitatively the rôle of the statocyst organs in geotropism. Such inquiries require a preliminary study of the conduct of *Uca* upon an inclined surface.

II.

The extreme sensitivity of the fiddler crab to light made it necessary to work in a dark room under red light. The eyes were covered with a mixture of lamp black and collodion as an additional precaution, since the animals frequently respond to "safe" light. The creeping plane was of wood, about 1 meter square, over which bolting cloth was glued to provide a surface favorable for creeping.

The direction of progression is sideways and in a straight line. Starting from the center of a horizontal line drawn at the base of the creeping plane, the entire path of progression was observed, for a length of about 50 cm., its direction fitted by a straight edge, and the angle measured with a protractor. Creeping toward right or left seemed to be random, and no attempt was made to control it. Five readings to right and five to left were taken for each animal at each value of α, occasional creepings to one side or the other not being recorded in order to maintain equality of numbers. Otherwise, all trails were recorded except those in which creeping became interrupted or disorganized, usually because of light penetrating an imperfect eye-covering, excessive handling, or slipping. Such individuals were replaced in the aquarium and used again only after an interval of several hours.

The angle α was set at random to inclinations of between 15° and 70°, at multiples of 5°. Below 15° readings were hopelessly inconsistent and above $\alpha = 70°$ the animals slipped. Table I gives the

values of Θ at $\alpha = 20°$ to 70° for one animal (No. 12, ♀) taken at random. The results with this individual are typical. The variation in Θ is quite small. As with other female fiddlers tested, there is no bias of magnitudes of response to right or to left. The agreement between values of Θ obtained from different individuals (♀ ♀) is

TABLE I.

Records of Angles of Upward Geotropic Orientation (θ) of Uca ♀ No. 12, at Different Slopes of the Surface (α).

α	20		25		30		35		40		45	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	30.7	29.5	45.0	45.2	43.2	34.5	39.4	37.2	57.5	48.6	53.8	64.4
	30.8	20.5	27.0	50.9	48.7	45.7	36.5	54.6	59.2	62.1	59.2	64.0
	28.3	30.7	42.6	26.2	38.2	45.6	42.0	49.8	52.0	46.3	69.8	57.4
	16.5	21.5	32.5	41.4	35.8	45.3	54.0	44.0	55.2	49.6	68.5	65.7
	18.2	32.0	44.5	37.8	40.6	42.4	50.0	55.0	51.8	46.3	62.3	66.3
		17.7										
Av. θ	24.90	24.24	38.32	40.3	41.30	42.70	44.38	48.12	55.14	50.58	62.72	63.56
Mean	24.57		39.31		42.00		46.25		52.86		63.14	

α	50		55		60		65		70	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	59.8	62.7	75.4	69.9	79.5	76.3	82.5	79.6	80.5	83.2
	69.8	65.4	69.8	79.9	73.6	78.2	76.6	80.4	84.1	81.0
	60.2	63.8	74.6	73.5	79.5	81.4	82.5	83.7	82.3	79.5
	65.5	70.4	75.7	72.1	74.8	78.7	77.8	81.7	79.0	83.6
	65.2	59.1	68.8	75.7	74.7	76.5	76.7	79.5	78.5	80.0
Av. θ	64.10	64.25	72.86	74.22	76.42	78.22	79.22	80.98	80.88	81.58
Mean	64.19		73.54		77.32		80.10		81.23	

shown in Fig. 1. That effects of handling were successfully eliminated is shown by the fact (*cf.* Crozier and Stier, 1927–28; 1928–29) that when the creeping platform was tilted after the animal had begun to move upon it, values of Θ were obtained agreeing precisely with those already recorded. Thus, with No. 9, tilting the surface to 30° gave a mean Θ of 39.59°; to 60°, Θ = 76.47 (*cf.* Table I).

III.

With female fiddlers the orientation angle Θ is the same regardless of the right or left direction of the path. For the males, normally asymmetric as regards the position of the enlarged chela, this is not the case (Table II; Fig. 6). The means of *right* and *left* values of Θ for

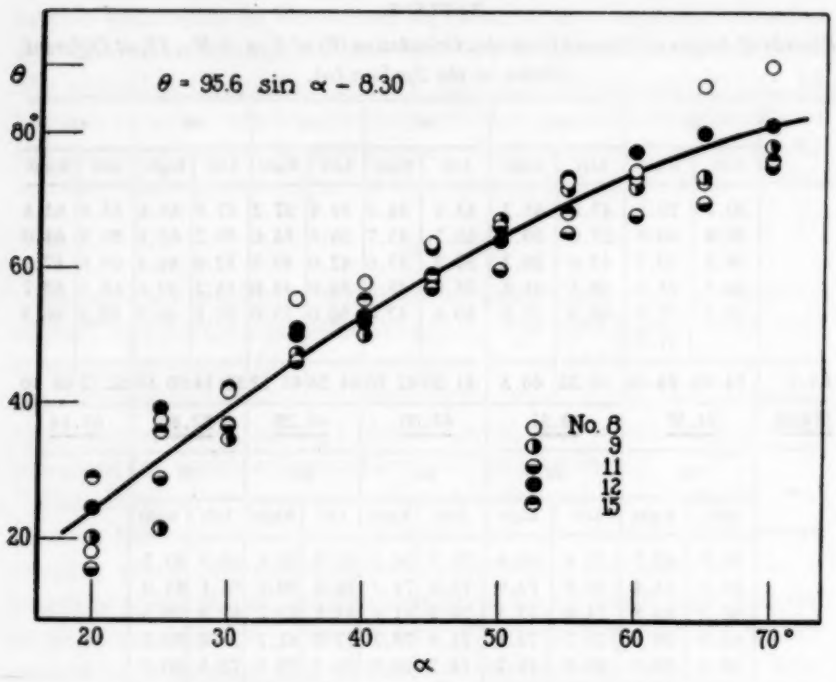

FIG. 1. Mean magnitudes of θ, the angle of the geotropically oriented path of progression, for female fiddler crabs on a surface with slope $\alpha°$; each point is the average of ten readings. The curve is transferred from that given in Fig. 2 as fitting the general averages.

the males, at each magnitude of α, agree very closely, however, with the corresponding mean Θ's obtained from the orientation paths of the females. The mean values of Θ are collected in Table II.

The relation of Θ to α is most simply expressed by a rectilinear connection of Θ with *sin* α (Fig. 2). A variety of other simple formulations fail to give an even approximately rectilinear plot. This case

is therefore similar to that of the beetle *Tetraopes* (Crozier and Stier, 1928–29), and with it differs sharply from various other instances[1] in which Θ is a linear function of *log sin α*.

In Fig. 2 the mean values of Θ have been plotted for females, and for males and females, the averages being calculated as means of the average orientations to right and to left.

The variability of the measured Θ's does not adhere to the same rules as found with young mammals.[1] The differing mechanics of the

TABLE II.

Mean values of orientation angle θ at different inclinations (α) of the surface on which *Uca* creeps, for (1) ♀ ♀ Nos. 8, 9, 11, 12, 15, moving to *right* and to *left*, (2) ♂ ♂ moving to *right* and to *left*, with their averages; and (3) for ♂ ♂ Nos. 10, 14, 16 with large claw on the left side, (4) ♂ ♂ Nos. 13, 17 with large claw on the right.

α	(1) θ, ♀ ♀			(2) θ, ♂ ♂			(3) θ, ♂ ♂ Nos. 10, 14, 16		(4) θ, ♂ ♂ Nos. 13, 17	
	Left	Right	Average	Left	Right	Average	Left	Right	Left	Right
20°	23.17°	19.78°	**21.48°**	23.26°	18.18°	**20.72°**	21.37°	19.49°	25.14°	16.87°
25	32.94	32.14	**32.58**	32.97	31.78	**32.38**	33.31	36.57	32.63	26.99
30	38.77	39.14	**38.96**	38.73	40.09	**39.41**	38.71	45.61	38.75	34.56
35	50.33	50.12	**50.23**	45.34	44.92	**45.14**	45.01	49.65	45.62	41.19
40	54.26	53.15	**53.70**	49.71	50.32	**50.02**	47.73	52.06	51.68	48.58
45	60.87	59.83	**60.34**	56.88	58.22	**57.55**	53.83	59.04	59.92	57.40
50	65.09	64.39	**64.75**	63.53	64.32	**63.76**	61.27	65.61	65.79	63.03
55	71.55	69.20	**70.38**	72.27	70.14	**71.21**	68.20	68.53	76.34	71.75
60	73.82	72.29	**73.06**	74.48	73.86	**74.17**	69.21	73.22	79.74	74.50
65	76.93	76.67	**77.26**	78.39	79.33	**79.22**	71.76	76.43	85.01	82.22
70	81.06	79.23	**80.15**	84.56	82.48	**83.62**	81.77	82.59	87.35	82.37

two cases leads one to expect such differences, and the mean Θ's have not quite the same significance. To a reasonable approximation, P. E.$_\theta$ here decreases linearly as *sin* α increases (Fig. 3). Such relationships provide an "internal" check on the validity of the measurements.

IV.

In contrast to the mode of creeping in most forms previously investigated for geotropic orientation, the body of *Uca* is not directly

supported on the surface of the inclined plane. A primary condition for stability will therefore be that the vertical line from the center of gravity of the animal must pass through the plane within the area

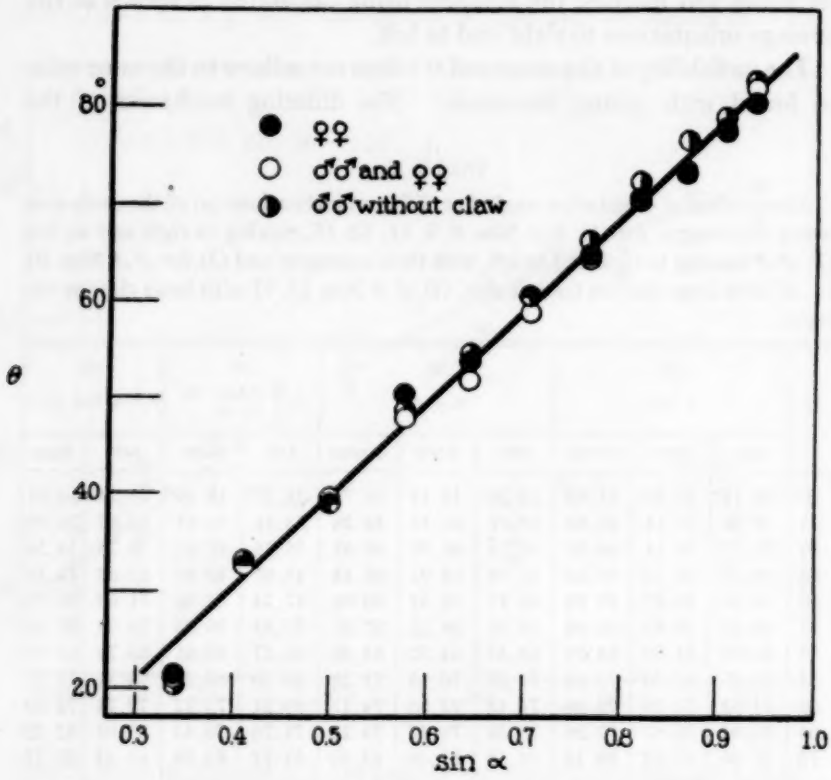

FIG. 2. Mean values of θ for ♀ ♀, and for ♀ ♀ and ♂ ♂, obtained as described in the text, give a rectilinear function with sin α. The same relationship holds for ♂ ♂ deprived of the large chela. The probable errors of the plotted points are less than 1° of arc.

delimited by lines connecting the points of support at the tips of the legs. An angle ρ may be defined as in Fig. 4, which is formed by the line connecting the tips of the most posterior legs with the intersection of creeping surface and the horizontal. Since direct observation shows

that the legs on the lower side of the crab are more widely extended, $\rho > \theta$, and we may write

$$\rho = \theta + m.$$

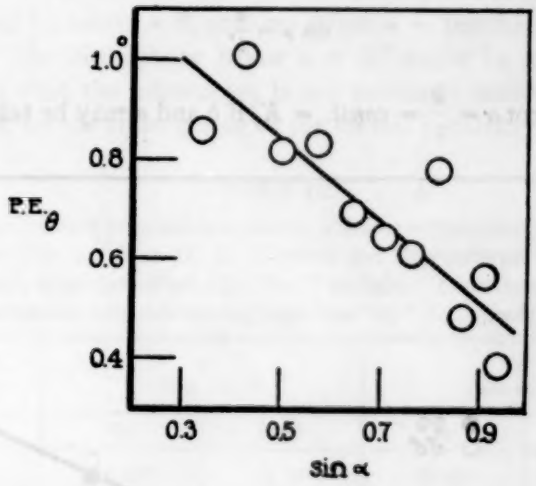

FIG. 3. The probable error of θ ($n = 50$) decreases in direct proportion to sin α.

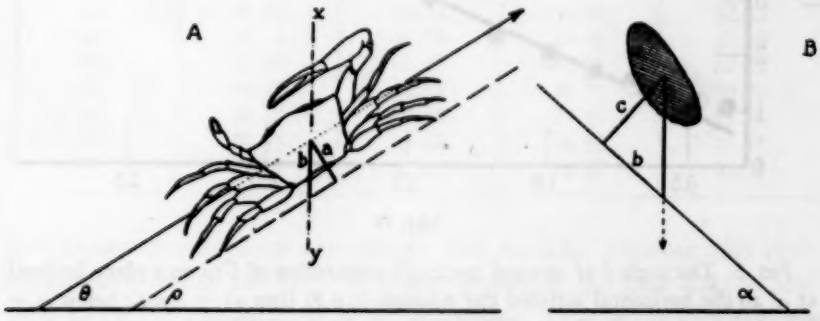

FIG. 4. *A*—Projection of *Uca* on the plane of creeping; direction of progression indicated by arrow. Diagramatic.
B—Section through the vertical plane x—·—·—y. (Different scale.) See text.

If the height of the body above the surface, in a direction normal to

the surface, may be taken as constant, so that c in Fig. 4 is constant, then the minimum condition for stability may be obtained thus:

$$\tan \alpha = \frac{b}{c}$$

$$\cos \rho = \frac{a}{b},$$

hence $\cos \rho / \cot \alpha = \frac{a}{c} = const. = K$, if b and a may be taken as con-

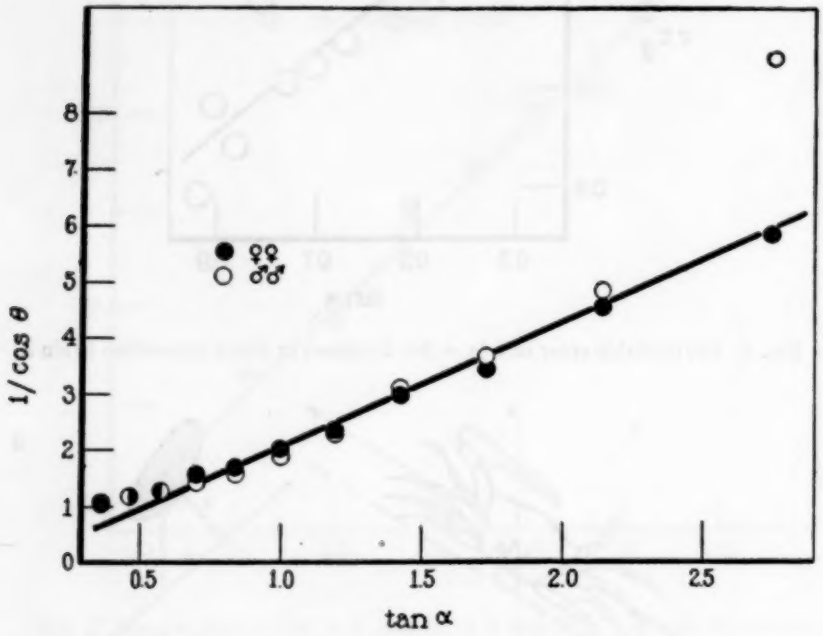

FIG. 5. The angle θ of upward geotropic orientation of *Uca* on a plane inclined at $\alpha°$ to the horizontal satisfied the relation $(\cos \theta)(\tan \alpha) = const.$; below $\alpha = 30°$, the apparent magnitude of θ is a little too high. Solid circles, mean θ's from measurements with ♀ ♀; open circles, ♂ ♂. The departure of the last point for the ♂ series may be due to slight slipping (2° of arc is enough to produce this departure). See text.

stant—*i.e.*, if the relative spread of the legs is the same at all values of α. Since $\rho = \Theta + m$,

$$\frac{\cos \theta \cos m + \sin \theta \sin m}{\cot \alpha} = K.$$

Taking data from the mean values of Θ for ♀ ♀, the responses for these being symmetrical, by substitution in this equation and trial it is found that computed values of K tend to pass through a maximum (although the variation is not great), unless m be made quite small. With $m = 2°$, K shows satisfactory constancy above $\alpha = 30°$. In fact, m may be taken = 0, and cos Θ/cot α = sensibly *const.* above $\alpha = 30°$. The discrepancy below $\alpha = 30°$ might be presumed due to the fact that the orientation is not primarily controlled, at low values of α, by the endangering of the animal's stability, or else that

TABLE III.

Mean magnitudes of orientation angle θ at different inclinations (α) of the creeping plane, for *Uca* ♂♂ Nos. 14, 16, 17 before and after removal of large chelæ. Nos. 14, 16 bore large claw on left side, No. 17 on right. Trails have been averaged on basis of movement with side bearing large claw "up" (*i.e.*, ahead) or down.

α	θ With chela		θ Chela removed	
	Up	Down	Up	Down
20°	18.60°	21.18°	20.43°	19.18°
25	31.49	34.11	32.45	33.78
30	37.71	44.35	42.10	37.90
35	43.06	49.25	50.74	46.60
40	49.00	53.54	56.07	52.62
45	55.12	61.00	63.08	57.65
50	63.90	66.85	66.87	67.14
55	70.21	70.94	74.11	70.25
60	68.78	75.40	77.11	76.51
65	76.25	79.45	80.85	76.77
70	84.05	84.63	84.47	84.57

the measurements here are simply too variable because the crab occasionally turns too far. It is also probably the case that at low values of α c is greater than at higher slopes, because the legs are less widely extended; this would produce the deviation as found. The result of this treatment is adequately shown by the plot in Fig. 5. The departures at $\alpha = 20°, 25°$ are within the errors of the measurements.[2]

[2] It might be argued that the posture of *Uca* on an inclined surface is determined simply by its behavior as a moving object on a rough plane. It is difficult to make

Satisfaction of the elementary conditions for mechanical stability means that a certain class of tensions on the legs is released, namely those tensions required to prevent the crab from toppling over. Hence

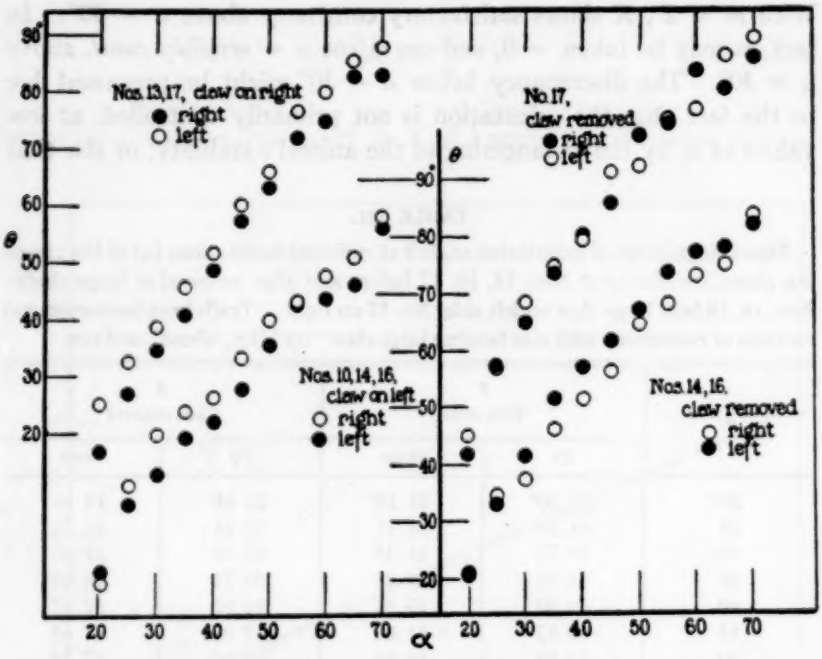

FIG. 6. Mean orientation angles to right and to left for 2 ♂♂ (13, 17) with large claw on the right side, and for 3 (10, 14, 16) with the large claw on the left, demonstrating lower θ's when creeping is toward the side carrying the large chela. This relationship is obliterated when the large claw is removed.

the result here obtained is in no sense a contradiction of the "muscle tension theory" of the control of geotropic orientation, but is merely an extension of it to a somewhat more complex case.

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refined application of this view, because of the nature of the crab's progression, but if we assume a "coefficient of friction" on the plane which acts normal to the axis of movement, then it can be shown that the condition for steady progression ought to be $\tan \theta / \tan \alpha = const.$ But this is not found. Moreover, this would leave unexplained the asymmetric response of normal males.

V.

The fact that the males of *Uca* characteristically bear one large and one small chela makes possible a further test of the muscle tension theory of geotropic orientation as applied to this case. Clearly, it would be expected, on the basis previously assumed, that consistently different values of the angle Θ should result depending upon whether the large claw is carried in advance, that is "up," or "down." If the center of gravity is shifted by the presence of the large claw, then Θ with the large claw "up" should be *less* than with this claw on the "down" side, regardless of whether the right or the left side bears the large claw. And the asymmetry of response should disappear if the claws are removed. In the case of female crabs no such difference should appear. The average Θ, from trails to the right and to the left, taken together, should moreover be about the same for males as for females. These expectations are in fact all realized.

The records were obtained in such a way that "right" and "left" trails were measured separately with each animal and the results kept distinct. The mean values of Θ for males with the large claw in the "up" posture and in the "down" (Table III) are plotted in Fig. 6. It is quite evident that Θ is consistently lower if carried "up," regardless of the right or left location of the large claw. The reason for this, it is supposed, is that in the males the center of gravity is shifted to one side and anteriorly by the weight of the enlarged chela. The males used weighed from 2.600 to 2.973 gm., their chelæ from 0.697 to 0.795 gm. So large a proportion of the total mass being located in the fiddle, the center of gravity must be more anterior than in the females. Hence, to obtain stability, it is not necessary to turn through so large an angle Θ when the claw is "up" as when it is on the "down" side (*cf.* Fig. 6), or in the absence of a large claw. (It has been pointed out previously that the mean of left and right Θ's is sensibly the same as with the females). If this be correct, the asymmetry evident in Fig. 6 should disappear with the removal of the claws of the males. Fig. 6 demonstrates that this expectation is in fact realized.

SUMMARY.

On an inclined surface the fiddler crab *Uca pugnax*, during sidewise progression, orients upward through an angle Θ on the surface. The

extent of negatively geotropic orientation (Θ) is a rectilinear function of *sin* α, where α is the inclination of the surface to the horizontal. This equation differs from that describing the geotropic orientation of various other animals. The difference is traced to the fact that from an initial position with the transverse axis of the body horizontal the crab is required to turn upward to an extent such that the vertical line from its center of gravity pierces the inclined surface within the base of support provided by the legs. This leads to the equation *sec* Θ/*tan* α = *const.*, which is obeyed within the limits of precision of the measurements. This type of control of geotropic orientation represents an extension of the "muscle tension theory," and is in no sense in conflict with this view. The assumptions underlying the analytical expression connecting Θ and α are verified by the asymmetry in the orientation of male fiddlers, which is shown to be due to the presence of the enlarged chela and which disappears when the claws are removed.

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SIGNIFICANCE OF THE CHEMICAL COMPOSITION OF THE SECRETING AND DRY MAMMARY GLAND TO MILK SECRETION.

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Of the constituents of milk, lactose is perhaps the most characteristic and least variable. A cow from the beginning to the end of her lactation or from lactation to lactation tends to produce a milk of relatively stable lactose content. Cows even of different breeds are much less divergent in the lactose percentages found in their milk than they are, for example, in the butter-fat percentages for the same milk. Lactose content has thus naturally become a starting point for the study of the mechanism of milk secretion. Gaines and Sanmann (1) have shown that the milk-secreting udder contains but negligible quantities of dextrose while large quantities of lactose are present. The amount of this lactose is closely related to the amount of milk which the udder is secreting at that particular time (1, 2) for if we determine the milk produced, its lactose per cent and total lactose, and compare this with the lactose content of the udder at a time corresponding to the normal milking period, the two lactose values closely agree as shown in Table I.

Table I shows that there is enough lactose present in the udder at the time of milking to more than account for the total amount of lactose produced by the cow in that milking. The average excess of lactose per cow is 0.0962 pounds. As the average lactose per cent of the milk drawn from these cows was 4.68, this difference represents an average equivalent of 2.1 pounds of milk remaining in the udder after the cow's udder is believed to be milked dry. The question naturally arises as to the justice of this comparison for it might be argued that the lactose represents a storage product to be drawn upon at some

subsequent lactation. That such is not the case is shown by the fact that the cow when dry, udder not secreting milk, has no reducing sugar in the udder tissue as shown in Table II.

The lactose of milk is believed to come from the dextrose of the blood (3, 4). Perhaps the best evidence for this conclusion comes from the analysis of the blood before entering the udder, generally

TABLE I.

*Total Lactose in Morning Milk Production and Total Lactose in the Udder at Time of Milk Production, Pounds.**

Cow No.	Lactose of milk production	Lactose in udder at time of milk production	Difference
	lbs.	*lbs.*	
137	0.129	0.206	0.077
97	0.187	0.353	0.166
114	0.194	0.315	0.121
221	0.333	0.323	−0.010
133	0.461	0.528	0.067
138	0.498	0.525	0.027
141	0.462	0.561	0.099
153	0.486	0.582	0.096
149	0.548	0.865	0.317
154	0.566	0.538	−0.028
111	0.596	0.718	0.122
1317A	0.639	0.784	0.145
148	0.649	0.614	−0.035
136	0.793	0.798	0.005
132	0.824	0.774	−0.050
124	0.856	0.931	0.075
144	0.876	1.090	0.214
1329A	1.035	1.288	0.253
1396A	1.227	1.313	0.086
5375A	1.264	1.441	0.177

* The customary unit of milk measure is the pound, 454 gm.

taken from the jugular vein, and blood leaving the udder, taken from the mammary vein. In the actively lactating udder the blood leaving the udder shows less dextrose than the simultaneously drawn blood of the general circulation. In the dry udder the blood of the general circulation shows no difference in its sugar content from that of the mammary vein. The general conclusion to be drawn from these facts,

i.e., that the lactose of milk is derived from the dextrose of the blood, is supported by the findings above as the udder in the lactating condition is shown to contain lactose whereas the dry udder does not.

TABLE II.

Lactose Found in Udder Tissue of Dry Cows.

Cow No.	Amount of lactose
118	None
155	"
167	"
170	"
44	"
61	"

TABLE III.

Chemical Content of Morning Milk and Udder with Contained Milk at Corresponding Times, Pounds.

Cow No.	Lactose		Fat		Ash		Nitrogen		Total solids	
	Milk	Udder	Milk	Udder	Milk	Udder	Milk	Udder	Milk	Udder
137	0.129	0.206	0.105	2.775	0.0230	0.1637	0.0218	0.2779	0.391	4.957
221	0.333	0.323	0.341	1.626	0.0494	0.1282	0.0414	0.2465	0.999	3.754
133	0.461	0.528	0.409	3.113	0.0684	0.1774	0.0532	0.3057	1.287	5.888
138	0.498	0.525	0.468	3.273	0.0905	0.2277	0.0649	0.3561	1.472	6.558
141	0.462	0.561	0.392	1.986	0.0890	0.6300	0.0720	0.4770	1.370	6.180
149	0.548	0.865	0.309	3.590	0.1060	0.3100	0.0690	0.4560	1.402	8.201
1317A	0.639	0.784	0.571	4.170	0.1060	0.6474	0.0802	0.5524	1.820	9.050
144	0.876	1.090	0.590	2.201	0.1530	0.3660	0.0930	0.5050	2.210	6.939
1329A	1.035	1.288	0.771	7.267	0.1624	0.5501	0.1299	0.9119	2.760	14.930
1396A	1.227	1.313	0.825	5.319	0.1628	0.4080	0.1137	0.6204	2.920	11.430
5375A	1.264	1.441	0.780	2.999	0.2018	0.4929	0.1264	0.7339	3.090	9.580

Chemical Composition of Milk as Related to the Chemical Composition of the Udder.

The chemical composition of the cow's udder when compared with the chemical composition of the milk produced throws some light on the raw materials stored by the udder for subsequent conversion into the constituents of milk. These data appear in Tables III and IV.

It is evident from an examination of Table III that at the time of milking there are sufficient materials in the udder to more than account for all the milk produced. This is particularly true of the fat, ash, and nitrogen. The lactose of the udder bears a much closer relation to the lactose of the milk than the other compounds of milk do to these same elements in the udder. This fact points to the conclusion that the lactose is formed from some raw product in the blood, probably dextrose, only as it is needed in the formation of milk. Fat, ash, and nitrogen have a fairly large excess reserve in the udder at all times. This is especially evident in the udder during the dry period as shown in Table IV.

TABLE IV.

Percentage Chemical Composition of the Udder during the Dry or Non-Lactating Period.

Cow No.	Lactose	Fat	Ash	Nitrogen	Total solids
44	None	17.37	0.79	2.28	34.8
61	"	18.42	0.73	2.39	34.2
118	"	40.49	0.59	1.64	52.1
155	"	29.87	0.78	1.67	44.8
167	"	72.08	0.23	0.93	78.5
170	"	60.91	0.34	1.16	69.5

The most noticeable changes in the composition of the dry udder are in the fat and the complete lack of lactose. The average composition of the udder tissue drained of milk is 13.3 per cent fat whereas the average composition of the dry udder tissue is 38.2 per cent. There is quite a wide variation in both groups, however, the composition of the drained udder tissue ranging from 7.6 to 20.7 per cent while that of the dry udder ranges from 18.4 to 72.1 per cent fat. The fat per cent of the secreting udder is significantly less than the dry udder even though this wide variation exists since for $n = 22$, $t = 5.2$ or P is much less than 0.01. These same relations also hold true for the water-free udder materials. The wide variation in both groups of data comes from the differences in the stages of lactation or gestation for the different cows. The fat of the dry udder is chemically different from butter-fat as the Reichert-Meissl number is practically 0 (0 to 0.4)

whereas that for butter-fat is larger than 24. The fat remaining in the udder after draining as much of the milk from the udder as possible approaches the Reichert-Meissl number of the dry udder. It seems unlikely that it would reach 0, however, as two cows which had the left side of the udder milked practically dry and then analyzed separately showed a Reichert-Meissl number of 11.8 and 14.4 respectively, whereas the right side of these udders containing 1 to 2 pounds more milk showed numbers of 11.1 and 13.0 respectively. The storage fat in the udder consequently appears different from butter-fat. The conversion of udder fat into butter-fat would therefore appear to progress somewhat faster than its utilization as in milk, a slight reserve being present.

Of reducing sugar the dry udder shows none whereas the udder in lactating condition contains its proportionate amount. The ash content of the dry udder shows a reduction to about half the amount found in the drained udder of the lactating cow (0.58 to 1.18 per cent). This difference is clearly significant as P is much less than 0.01. The nitrogen is practically the same in the dry udder as it is in the udder drained of milk, 1.66 to 1.79 per cent. The dry or quiescent mammary gland builds up a fat reserve of different Reichert-Meissl number than butter-fat, secrets no sugar, reduces its ash content, and has a nitrogen content practically that of the actively secreting udder.

SUMMARY.

The results herein presented furnish exact critical evidence for one more stage in milk secretion. Cows producing up to 30 pounds of milk at one milking are shown to have the lactose equivalent of all this milk in the udder when milking commences. The average excess of lactose found in the udder after subtracting the amount necessary for the contained milk is equal to 2.1 pounds. This represents the milk retained in the udder when the cow is believed to be dry. These conclusions are further supported by the fact that no sugar is found in the udder in the quiescent state.

The study of the total composition of the udder as fat, ash, nitrogen, and lactose, and of the contained milk shows that there is a large excess of fat, ash, and nitrogen in proportion to that necessary for milk formation. The excess of udder lactose over the milk lactose is much less.

The lactose would therefore appear to be formed from some element in the blood, probably dextrose, only as needed for the formation of milk.

The composition of the dry udder is quite different in certain respects from that of the actively secreting gland. It builds up a fat reserve of a quite different Reichert-Meissl number from that of butter-fat. It has no sugar, its ash content is reduced, and the nitrogen content is like that of the secreting gland.

Technique.—The method of extracting the lactose of Cows 97, 111, 114, 124, 132, 136, 148, 153, and 154 is described in (2). This method has been somewhat improved for the other cows indicated. The morning milk yield was determined as in the past. The cow was killed as before at the time when she had previously been milked for the morning milk yield. The udder and contained milk was then dissected off and separated into its two halves. The analysis was made separately on the two halves. The half to be analyzed was freed of any skin and teats, weighed and cut into inch cubes. The milk which drained out was caught and analyzed. The inch cubes were ground through a power meat grinder, passing through a plate carrying 1/16 inch holes. This material when well mixed was sampled and analyzed. Emphasis should be laid on the fact that this analysis should be made with as little delay as possible. The milk and drained milk from the udder were analyzed by the ordinary milk technique. The ground material was extracted for lactose, the lactose being determined with the polariscope. The material was dried to constant weight and extracted for the fat. The material was ashed in an electric oven. The nitrogen was determined by the Kjeldahl method.

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THE EFFECT OF CERTAIN ELECTROLYTES AND NON-ELECTROLYTES ON PERMEABILITY OF LIVING CELLS TO WATER.

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Although permeability to water must be regarded as one of the most fundamental properties of living cells, the degree of permeability may be surprisingly low (1), even temporarily nil (2) and varies with the physiological state of the cell (3). It is also thought to depend on the chemical composition of the medium, but direct evidence on this point is scanty. Experiments were therefore undertaken to show quantitatively whether cell permeability to water is regulated by electrolytes in the medium.

A satisfactory method for attacking this problem is to place the cell in anisotonic solutions, so causing water to enter or leave the cell under the driving force of osmotic pressure. The unfertilized egg of the sea urchin, *Arbacia punctulata*, is an excellent natural osmometer for this purpose; the amount of swelling or shrinking occurring in anisotonic solutions, and hence the volume of water entering or leaving the cell, can readily be measured under the microscope.

The effect of temperature and of the salt concentration of the medium on the rate of this process has been previously reported (4–6). In the present experiments, these factors have been held constant, and only the chemical composition of the medium varied. Under these conditions, change in rate of osmosis may be interpreted as change in permeability of the cell to water.

By permeability is understood the quantity of material (in this case, water) passing through unit area and unit thickness[1] of membrane in unit time under unit pressure. In order that results may be expressed

[1] We have assumed that the thickness of the membrane is constant.

in dimensions of permeability, the empirical equation[2] employed in previous papers has not been used here, although the velocity constant

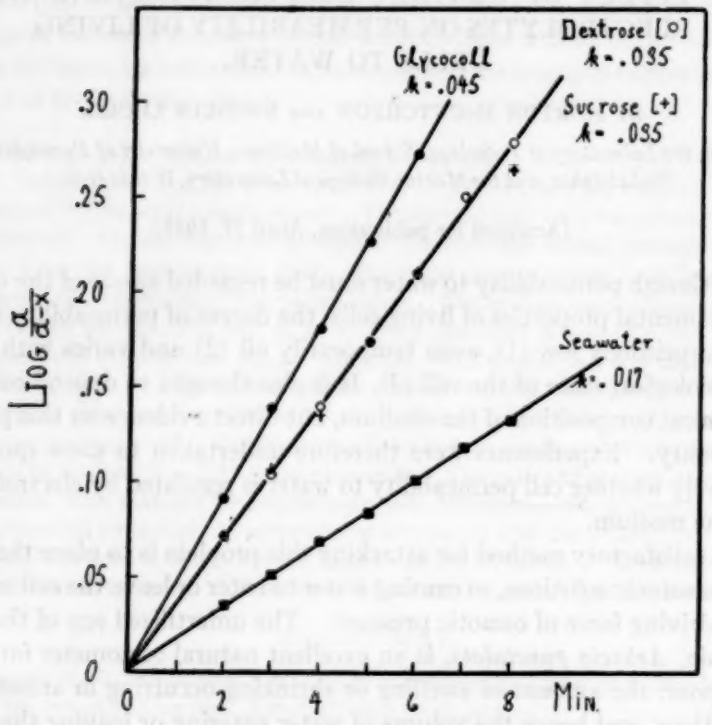

FIG. 1. The effect of non-electrolytes on rate of swelling in hypotonic solutions. Data are taken from Table I. When $\log \frac{a}{a-x}$ (in which a is total increase in cell volume, and $a - x$ is volume increase up to time t) is plotted against times, the graph is a straight line, showing that the process follows the equation $kt = ln \frac{a}{a-x}$, in which k is the velocity constant.

of that equation is approximately proportional to the permeability, provided that, as here, temperature and osmotic pressure of the

[2] $\frac{dx}{dt} = k(a - x)$, where a is the total volume of water that will cross the membrane before equilibrium is established, x the amount that has already crossed at time t and k is the velocity constant.

medium are not varied. The empirical equation fits the present data, as shown in Fig. 1.

In this paper, results are calculated from the equation, permeability $= \frac{dV}{dt} \Big/ SP$ in which $\frac{dV}{dt}$ is cubic micra of water per minute, S is square micra of surface and P is atmospheres.

From the data in the accompanying tables, cell volumes were plotted against times, and a smooth curve drawn through the points. The rate of endosmosis was determined in each instance at the *second minute*, since earlier observations were frequently impossible on account of the time required for cells to settle. The rate at the second minute may be determined by drawing the tangent to the curve at this point, but since it is difficult to draw tangents accurately, it was found more satisfactory to determine the times required, beginning at the second minute, for the cells to increase 20,000 cubic micra in volume. The assumption made, that this portion of the curve is a straight line, while not strictly correct, introduces no serious error.

From the figure so obtained, increase in volume per minute was calculated. Practically identical rates were found by drawing tangents.

Surface and pressure were calculated for a point midway between the volume at 2 minutes and a volume greater by 20,000 cubic micra, *i.e.* volume at 2 minutes plus 10,000 cubic micra. Surface was calculated directly from the volume read from the curve at this point. Pressure inside the cell was calculated from the equation $P_t = P_o V_o / V_t$ in which P_o and P_t are osmotic pressures inside the cell at the first instant and at time t respectively, and V_o and V_t are the corresponding cell volumes. P_o was taken as 22 atmospheres, V_o was taken as the mean volume of 30 eggs of the same animal measured in sea water, V_t was read from the curve and P_t was calculated. The osmotic driving force is the difference between P_t and the pressure of the solution -8.8 atmospheres for 40 per cent sea water. The values so obtained were substituted in the expression for permeability.

The technic of measuring volume changes in *Arbacia* eggs by means of a filar micrometer eyepiece has been previously described (4). In the experiments here reported, the cells were first washed in isotonic concentration of the solution to be employed, to remove electrolytes.

A drop of cell suspension was thoroughly mixed with about 20 cc. of this solution, and the cells were then allowed to settle. Temperature was maintained by a water jacket at 12 ± 0.5°C.[3]

Effect of Non-Electrolytes.

The first step, preparatory to studying the effect of electrolytes, was to eliminate them from the medium and to learn whether the rate with

TABLE I.

The effect of non-electrolytes, contrasted with that of sea water, on the permeability of unfertilized *Arbacia* eggs to water. Solutions are isosmotic with 40 per cent sea water. Cell volumes are given in cubic micra $\times 10^2$. Each number represents the mean volume of 6 cells. In the bottom row is given the permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was 12°C. (data are graphed in Fig. 1).

It is seen that permeability in non-electrolyte solutions is much higher than in sea water.

The mean volume of 30 control cells in 100 per cent sea water was 2060×10^2.

Time	Glycocoll	Dextrose	Saccharose	Sea water
m/s.				
2	2475	2325	2410	2265
3	2710	2490	2595	2355
4	2900	2675	2725	2450
5	3075	2830	2900	2525
6	3250	2980	3050	2620
7		3145		2700
8		3245		2760
Permeability	0.142	0.097	0.103	0.050

which water entered the cells from hypotonic solutions was thereby affected. Accordingly cells were placed in hypotonic aqueous solutions of non-electrolytes—dextrose and saccharose, and also of glycocoll, which, on account of its slight electrolytic dissociation may be expected to behave toward cells like a non-electrolyte.[4]

[3] Low temperature was necessary to prevent excessively rapid swelling in some of the solutions.

[4] In most experiments solutions were made isotonic with 40 per cent sea water (sea water 40 parts, distilled water 60 parts). At Woods Hole this solution has,

The effect of these non-electrolytes is illustrated in Table I. It is seen that permeability to water is much greater in these solutions than in sea water of the same osmotic pressure (in most experiments it was about twice as great). Other experiments showed the differences in permeability in dextrose, saccharose and glycocoll to be probably not significant.[5]

A possible objection to the significance of these results is that the hydrogen ion concentration was not the same in the several solutions, dextrose, saccharose and glycocoll being on the acid side of neutrality, sea water on the alkaline. This objection is believed not to be valid, as preliminary experiments showed that increase in hydrogen ion concentration obtained by adding HCl to dextrose solution tends to decrease permeability.

Therefore, the rapid osmosis observed in non-electrolyte solutions is not due to increased hydrogen ion concentration, but either to the effect of non-electrolytes or to the absence of electrolytes.

Effect of Sodium and Potassium.

Supposing that the effect of non-electrolyte solutions were due to absence of electrolytes, it seemed possible that addition of electrolytes, especially of those occurring in sea water, to sugar solutions might lower permeability to that observed in sea water. All electrolytes might have this effect or only certain ones. Accordingly NaCl, which is the most abundant electrolyte in sea water, was added to hypotonic dextrose solution; the concentration of NaCl was 0.01 molar.[6]

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according to Garrey (7), a freezing point of 0.73°C. Calculations based on data in the Landolt-Börnstein Tabellen show that 0.38 molar dextrose or 0.39 molar saccharose should be isotonic with this solution. 0.4 molar was assumed as approximately the isotonic concentration of glycocoll.

[5] Samples of dextrose from three manufacturers gave similar results. It is assumed that no significant penetration of these substances occurred during the short time that experiments lasted.

These non-electrolytes were selected because they are known not to penetrate cells readily. The choice of non-electrolytes is important because if substances were used which rapidly enter the cell, its osmotic pressure would increase and endosmosis would be accelerated.

[6] Dextrose was chosen as the most convenient non-electrolyte with which to work.

Electrolytes were dissolved in distilled water in 1 molar concentration, the

The effect of NaCl and of KCl is illustrated in Table II. It is seen that the permeability values were even greater than in solutions of dextrose alone. Repeated experiments showed that there is probably no significant difference in the effect of Na and K, nor did these ions

TABLE II.

The effect of sodium, potassium, calcium and magnesium on permeability to water. The chloride of the several cations is added to dextrose in amounts sufficient to give 0.004 molar concentration of the electrolytes. Solutions are isosmotic with 40 per cent sea water. Each number represents the mean volume of 6 to 9 cells.

It is seen that in NaCl and KCl, permeability to water is of the same order of magnitude as in dextrose alone, while in $CaCl_2$ and $MgCl_2$ it is approximately the same as in sea water.

The mean volume of 30 control cells in 100 per cent sea water was 2035×10^3.

Time	Dextrose	NaCl in dextrose	KCl in dextrose	$CaCl_2$ in dextrose	$MgCl_2$ in dextrose	Sea water
min.						
1						2210
1.5		2205				
2	2180	2310	2290	2245	2240	2285
2.5		2435				
3	2305	2560	2470	2365	2365	2370
3.5		2655				
4	2480		2580	2460	2435	2465
4.5		2900				
5	2655		2750	2535	2520	2545
6	2840		2855	2610	2595	2630
7	2955			2685	2635	2685
8	3070			2745		2740
Permeability	0.093	0.129	0.096	0.054	0.050	0.048

invariably increase the rate of osmosis above that occurring in solution of dextrose alone. Lower concentrations of NaCl and KCl produced similar though less definite results; higher concentrations led to rapid cytolysis.

amounts of these solutions added to dextrose being so small as not to alter its osmotic pressure significantly.

Effect of Calcium and Magnesium.

There remained the possibility that only certain of the electrolytes of sea water have a restraining action on osmosis. Since Na and K tended to increase permeability to water, it seemed possible that Ca and Mg would have the opposite effect. This proved to be the case. In the same Table (II), the value in hypotonic sea water is 0.048, in dextrose solution of the same osmotic pressure, 0.093. On adding $CaCl_2$ to this dextrose solution in amount sufficient to obtain 0.004 molar concentration, the permeability was reduced to 0.054, approximately the value in sea water. This result was regularly reproducible. A similar effect was produced by $MgCl_2$.

Remarkably small amounts of Ca were found effective in slowing osmosis in dextrose, though a concentration of 0.00005 molar proved to be too low. Thus in one experiment in which the concentration of $CaCl_2$ was varied, the following results were obtained: 0.001 M, 0.044; 0.0005 M, 0.042; 0.0001 M, 0.049; 0.00005 M, 0.077.

These experiments indicate that the rapid osmosis observed in dextrose solutions is not due to the effect of non-electrolytes, but to absence of Ca and Mg. Also, the conclusion appears justified, that the relatively slow osmosis observed in sea water is due, at least in part, to the presence of these two bivalent cations.

It has been seen that the chlorides of Ca and Mg in dextrose solutions decrease permeability to the value found in sea water, whereas NaCl and KCl have no such effect. Numerous experiments invariably gave similar results.

Antagonism of NaCl and KCl with $CaCl_2$ and $MgCl_2$.

As stated above, Na and K in dextrose solution did not always increase permeability more than did dextrose alone. In order to determine the effect of these cations more definitely the principle of salt antagonism was used. Having found a concentration of $CaCl_2$ in dextrose solution which was just sufficient to lower permeability to the value obtained in sea water, varying amounts of NaCl were added. In appropriate concentrations, NaCl antagonised the slowing effect of $CaCl_2$, the degree of permeability being intermediate in value between that of Ca in dextrose and that in dextrose alone. K was just as effective as Na in this respect. A typical experiment is represented in

Table III. Ca could be replaced by Mg and similar antagonism with the univalent cations demonstrated. Such experiments show definitely that NaCl and KCl tend to increase permeability to water.

Preliminary experiments indicate that this method of studying salt antagonism can also be used quantitatively. Table IV shows that increasing amounts of NaCl added to $CaCl_2$ in dextrose give increasing values for permeability.

TABLE III.

Antagonism of sodium or potassium with calcium. In 0.0005 molar $CaCl_2$ in dextrose, permeability to water has the same low value as is usually obtained in isosmotic (40 per cent) sea water. Upon the further addition of NaCl or KCl (final concentration 0.01 molar), permeability increases to values intermediate between those in calcium-dextrose solution and in dextrose alone.

Each figure represents the mean volume of 5 to 7 cells. Volume of control in 100 per cent sea water, 2030×10^2.

Time	Dextrose	KCl and $CaCl_2$ in dextrose	NaCl and $CaCl_2$ in dextrose	$CaCl_2$ in dextrose
min.				
1		2170	2015	2195
2	2285	2325	2145	2255
3	2470	2435	2295	2355
4	2595	2585	2405	2450
5	2735	2695	2520	2520
6	2890	2820	2645	2580
7	2995	2935	2770	2650
8		3045	2850	2720
Permeability	0.091	0.076	0.065	0.050

DISCUSSION.

These experiments indicate that the cations of sea water are important in regulating the permeability of the cell to water. There is an extensive literature on the effect of ions on permeability of cells and tissues to various substances.[7] In most cases, as in our experiments

[7] The literature on this subject is reviewed by Osterhout, W. J. V., Injury, recovery, and death in relation to conductivity and permeability, Philadelphia and London, 1922. Jacobs, M. H., in Cowdry, E. V., General cytology, Chicago, 1924. Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926. von Tschermak, A., Allgemeine Physiologie. I, Berlin, 1924.

given above, Na and K have been found to increase permeability, Ca and Mg to decrease it. Particularly striking is the agreement between our results and those of Osterhout (8), who studied the effect of various electrolytes on the electrical conductivity of plant tissues, using this property as a measure of permeability. For these reasons it is probable that the action of ions stated above has general application.

TABLE IV.

Antagonism of sodium and calcium. Having determined permeability to water in solution of 0.0005 molar $CaCl_2$ in 0.38 molar dextrose, increasing amounts of NaCl are added to this solution. Permeability is seen to increase with the amount of NaCl added. Each figure represents the mean volume of 5 or 6 cells. The mean volume of 30 control cells in 100 per cent sea water was 2010×10^2.

Time	$CaCl_2$ and 0.016 M NaCl	$CaCl_2$ and 0.008 M NaCl	$CaCl_2$ and 0.004M NaCl	$CaCl_2$
min.				
1	2115			
1.5	2180			
2	2270	2245	2170	2230
3		2330	2265	2325
4	2525		2385	2420
5	2675	2520	2430	2480
6	2830	2640	2535	2545
7		2760	2675	2620
8		2890	2765	2710
Permeability	0.080	0.056	0.050	0.047

On the other hand the effect of non-electrolytes seems to vary with the material and method used (9).

The method employed by us permits direct measurement of the rate with which water crosses the cell membrane. The effect of different ions on permeability is striking and reproducible. Since the experiments were carried out at constant temperature and constant osmotic pressure of the medium, the conclusion is justified that the permeability of the cell to water is increased by NaCl and KCl, decreased by $CaCl_2$ and $MgCl_2$.[8]

[8] The *mechanism* of these changes in permeability to water has not been investigated in the present series of experiments. It may be said, however, that injury

SUMMARY.

1. Permeability to water in unfertilized eggs of the sea urchin, *Arbacia punctulata*, is found to be greater in hypotonic solutions of dextrose, saccharose and glycocoll than in sea water of the same osmotic pressure.

2. The addition to dextrose solution of small amounts of $CaCl_2$ or $MgCl_2$ restores the permeability approximately to the value obtained in sea water.

3. This effect of $CaCl_2$ and $MgCl_2$ is antagonized by the further addition of NaCl or KCl.

4. It is concluded that the NaCl and KCl tend to increase the permeability of the cell to water, $CaCl_2$ and $MgCl_2$ to decrease it.

5. The method here employed can be used for quantitative study of salt antagonism.

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9. Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926, 490, 563.

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to the cell is probably involved. Preliminary experiments of various types—such as fertilization tests on cells previously exposed for a few minutes to isotonic unbalanced solutions—suggest that NaCl and KCl are more toxic than $CaCl_2$ and $MgCl_2$, and that recovery from injury may be possible to a certain degree. But on all these points more data are necessary before definite conclusions are drawn. It would be of considerable importance if it could be shown that under certain conditions, increase in permeability of the cell to water could be taken as a measure of injury.

OXIDATION-REDUCTION EQUILIBRIA IN BIOLOGICAL SYSTEMS.

I. Reduction Potentials of Sterile Culture Bouillon.

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(Accepted for publication, April 27, 1928.)

Oxidation-reduction reactions play an important rôle in the metabolism of all living cells. Since such reactions are often brought about in the medium outside of the living cell, a consideration of the oxidation-reduction equilibrium in sterile culture bouillon has seemed desirable because of its possible significance for the reductions manifested by bacteria in culture.

The only previous study of this problem was made by Theobald Smith,[1] who found that the reduction of methylene blue may be brought about by sterile sugar-free bouillon in the closed arm of the fermentation tube; in the presence of dextrose the reduction also of litmus takes place. These observations of Smith appear not to be generally known; they have not received the extension which they appear to warrant possibly because of the lack of suitable indicator substances and the want of theory covering their action as indicators of reduction.

Although the recent work of Clark[2] and his collaborators has met both of these deficiencies the electrometric measurement of oxidation-reduction equilibria provides a method which makes possible consideration of both the intensity and the capacity factors of reaction, and permits the statement of results in more precise terms than those of dye reduction. These considerations are peculiarly applicable to a poorly poised system such as culture bouillon, and experience in the

[1] Smith, T., *Cent. Bakt., 1. Abt.*, 1896, xix, 181.

[2] Clark, W. M., *Pub. Health Reports*, 1926, suppl. 55, and earlier papers to which reference is given here.

preliminary work of the present investigation has shown the advantage of the electrometric method. Justification may be required for the use of this method in biological systems in which the essential reversibility of the reactions involved has not been established and in which the reacting substances have not been identified. The validity of the application is being established however by the work of recent investigators.[2–6] The significance of electrode potentials for the state of reduction of a system has been discussed by Clark[7] and Conant[8] and need not be undertaken here.

Technic.

In the determination of reduction potentials in the present work attention has been limited, in order to simplify conditions, to bouillon prepared without the addition of carbohydrate. It was made in the usual way with meat-infusion. The pH was adjusted to 7.6 and stabilized by the addition of phosphate buffer to a final concentration of M/15, except in a few of the earlier experiments in which phosphate was not added. Sterilization was effected in an Arnold sterilizer, or in the autoclave at 10 lbs. for 10 minutes. In all of the later experiments the bouillon was autoclaved as described immediately before the experiment was started. The native dextrose of the medium was not fermented out.

The technic of measurement of the reduction potentials has been similar to that commonly employed with special attention to the purification of the metal used for the electrode. The electrode cell was either a weighing bottle, 4×5 cm. in dimensions, or a special vessel of similar size provided with a bottom outlet closed by a stop-cock. This was designed particularly for work to be reported on in a later paper. The mouth of the vessel was closed by a No. 9 rubber stopper, containing one central and eight peripheral perforations. These served for the introduction of (1) a separatory funnel through which solutions could be introduced into the cell, (2) the nitrogen inlet and outlet tubes, (3) the KCl-agar bridge tube, (4) a thermometer, and (5) four gold electrodes. All the glass tubes including that for the salt bridge were provided with stop-cocks which were lubricated with a mixture of paraffin and petrolatum. The agar bridge and the thermometer were sterilized separately and then fitted aseptically to the rest of the apparatus which had been assembled and autoclaved.

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[3] LaMer, V. K., and Baker, L. E., *J. Am. Chem. Soc.*, 1922, xliv, 1954.

[4] Conant, J. B., and Fieser, L. F., *J. Biol. Chem.*, 1924–25, lxii, 595.

[5] Dixon, M., and Quastel, J. H., *J. Chem. Soc.*, 1923, cxxiii, 2943.

[6] Kenny, C. L., Dissertation, Columbia University, 1926.
LaMer, V. K., Kenny, C. L., and Sherman, H. C., to be published.

[7] Clark, W. M., *Chem. Rev.*, 1925, ii, 127.

[8] Conant, J. B., *Chem. Rev.*, 1926, iii, 1.

Nitrogen for deaeration was drawn from a commercial cylinder and allowed to pass through copper tubing filled with tightly rolled copper gauze. The only rubber tubing in the system was the short length which connected the copper tubing with the nitrogen inlet of the cell. The exposed surfaces of this rubber tube and of the rubber stopper were shellacked. The copper tubing with enclosed gauze was heated at its middle by an electrical coil or a large Bunsen flame and was cooled near the electrode cell by a water jacket. The nitrogen inlet tube of the cell was drawn out to a point with an internal diameter of 0.5 mm., and was placed so as to reach almost to the bottom of the cell, so that with the flow of nitrogen a stream of very small bubbles passed through the bouillon and assisted in keeping it agitated. No other stirring device was used, as necessity for it did not appear.

The preparation of the gold which was used as the electrode metal received the most careful attention. It was found early in the investigation that close agreement between different electrodes in the same solution could not be secured with the purest gold commercially obtainable. Consequently gold was purified first in solution and then as metal by cupellation with borax on a graphite cupel, in order to remove as far as possible traces of less noble metals and of silica. The solubility of silica in gold is worthy of remark. The purified gold button was fused to the purest platinum wire which could be obtained; this was sealed into glass tubing. Only those electrodes so prepared were used which were found equipotential when tested in ferri-ferro-cyanide solution and were equipotential or in very close agreement in bouillon exposed to air. I am indebted to Professor LaMer for this method of testing the electrodes.

A sensitive galvanometer was used as the null-point instrument. The advantage of an electrometer has not been apparent for this particular investigation. It has frequently been found necessary to render electrodes sensitive by slight polarization; any reversible change produced thereby in the system is reversed readily by alteration of polarity, and only such reversible reactions can be regarded as significant in the measurement of potentials. The potentials observed have been referred to the normal hydrogen electrode to give Eh values for pH 7.6 which was maintained in the bouillon throughout the series of experiments.

The reduction potentials of sterile culture bouillon are given in the form of time-potential curves. The initial potentials are variable and are poorly poised; in different experiments they have been found to lie between + 0.250 and + 0.150 volt. Individual electrodes during the first part of the curve may be in disagreement by as much as 50 millivolts, but have often been found to agree within 5 millivolts when the electrode metal has been purified in the manner described. Succeeding the irregular initial potentials is observed a steady negative drift or trend toward a region of greater reduction intensity.

The rate of this drift depends, with other factors, upon the rapidity with which oxygen is swept out of the system. If the exposed surfaces of rubber are shellacked, and if diffusion of oxygen through the cocks in the various tubes of the apparatus is prevented by careful fitting and lubrication, the oxygen is removed at the maximum rate. The potential then falls as shown for typical experiments with two different specimens of bouillon in Fig. 1, and within 5 or 6 hours

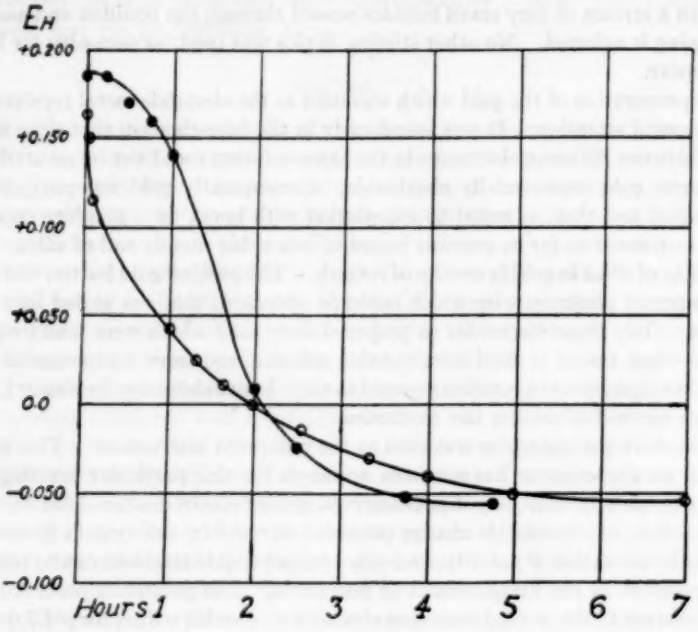

FIG. 1. Time potential curves of two specimens of sterile bouillon. Deaeration was started at the beginning of potential measurements and was continued throughout.

approaches a value between − 0.050 and − 0.060 volt. This appears to be a limiting value for the majority of lots of bouillon examined, and may be regarded as a characteristic potential for the system. After the technic had been fully developed and proper attention paid to exhaustion of oxygen from the nitrogen and to complete sealing of the apparatus against diffusion of oxygen from the outside, it was possible to duplicate results almost exactly with a

given lot of bouillon. The limiting value for the potentials and the time required to reach this have been very nearly the same for specimens of bouillon prepared at different times, when the same perfected technic was employed, although the configuration of the potential curve varies slightly.

Another observation has been made which is of considerable interest and is essentially a confirmation of the findings of Theobald Smith. In the earlier experiments in which exclusion of oxygen from the

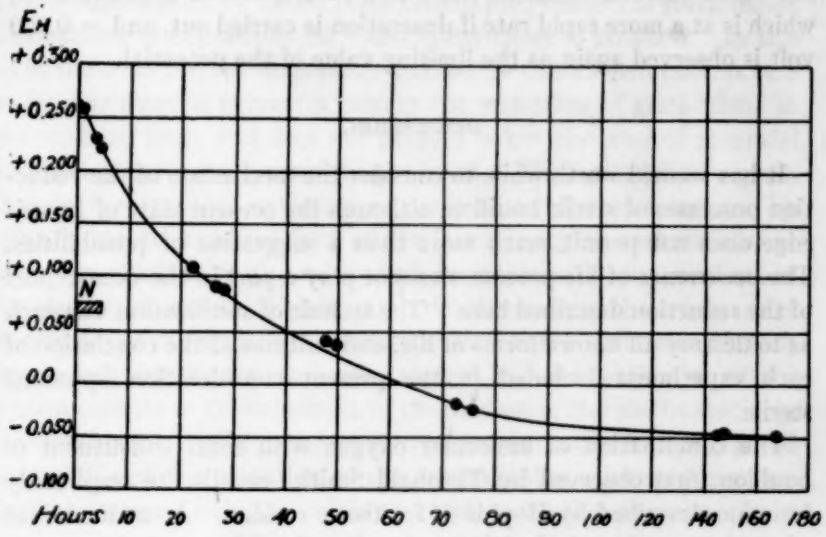

FIG. 2. Time potential curve of sterile bouillon, deaerated during the first 5 hours only of potential measurement.

system was not so complete as in the later ones, the potential did not reach the limiting value after 4 to 6 hours deaeration. If at this time the flow of nitrogen is discontinued and the vessel sealed against the entrance of oxygen by shellacking the rubber surfaces and covering stop-cocks with petrolatum, the potential continues to fall slowly and after 5 days or longer attains the limiting value above noted of −0.060 to − 0.050 volt. The conditions are then similar to those studied by Theobald Smith: the oxygen in the bouillon within the closed arm of the fermentation tube becomes exhausted and the indicator dye is

reduced. Fig. 2 shows the course of potentials which corresponds to these events, although here no dye was present and a mechanism similar to that suggested by Kendall[9] could not be involved.

If oxygen is admitted into the system after the potentials have begun to decline from the initial points or have reached the limiting value, the electrodes record an immediate shift to more positive values and may return to those initially observed if the aeration be sufficient. Renewed deaeration, or merely sealing of the apparatus against diffusion where this has occurred leads to a resumption of negative drift, which is at a more rapid rate if deaeration is carried out, and − 0.060 volt is observed again as the limiting value of the potential.

DISCUSSION.

It has seemed worth while to consider the mechanism of the reduction processes of sterile bouillon, although the present state of knowledge does not permit much more than a suggestion of possibilities. The occurrence of life processes cannot play a part in the occurrences of the reduction described here. The technic of sterilization was such as to destroy all known forms of life, and cultures at the conclusion of each experiment included in the present consideration appeared sterile.

The combination of molecular oxygen with some constituent of bouillon, first observed by Theobald Smith,[1] recalls the respiratory function described by Hopkins[10] for tissue residue. A small amount of a substance identical with or related to the water-insoluble muscle residue may be present in bouillon, contributing perhaps to its colloidal character. Bouillon contains glutathione extracted from chopped meat, and there may occur an action similar to that which Hopkins has described, but at a slow rate, in which glutathione acts as a catalyst in bringing about oxidation of the muscle residue by molecular oxygen. There is evident in any case the existence in bouillon of some autoxidizable substance the oxidation of which is accompanied by the reduction of electromotively active substances such as glutathione or indicator dye; the latter substances must be regarded as responsible

[9] Kendall, E. C., *Science*, 1928, lxvii, 379.
[10] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

for the potentials observed. The autoxidizable substance may undergo irreversible oxidation like many organic compounds, and would therefore be expected to give by itself no measurable oxidation potential.

It is significant that the limiting value of negative potential manifested by sterile bouillon of pH 7.6 should be so nearly the same as that observed by Dixon and Quastel[6] in a solution of reduced glutathione and found by Clark[2] to be the equilibrium value in the reduction brought about by boiled yeast cells in the presence of glutathione. Glutathione is apparently an active constituent of bouillon. In this as in the reducing cell suspension studied by Clark,[2] reduction occurs as long as there is present a certain concentration of glutathione in the oxidized form, and does not proceed below the level of potential at which glutathione is completely reduced. The limited data suggest that in the catalytic oxidation of some autoxidizable substance by glutathione, the oxidized form of the latter is active, and the change from the disulfide to the sulfhydryl arrangement is the mechanism for electron transfer. This is only a development of the argument of Hopkins, but it emphasizes the idea that it is the glutathione which is primarily oxidized by molecular oxygen and that activation of the system consists in the formation of the oxidant of the electromotively active substance. This involves the conclusion that both the observed potentials and the oxidation of the autoxidizable substance measure the free energy of reduction of the glutathione or other electromotively active substance and implies that reduction does not proceed to a more negative level *because* there is no system present which could yield measurable potentials.

The capacity of bouillon to combine with oxygen as shown by the continued fall in potential after partial deaeration is not exhausted by standing for 9 months freely exposed to air in Erlenmeyer flasks, or as in Theobald Smith's experiments after several successive aerations and subsequent reductions of methylene blue. Consideration of other factors than those discussed above may therefore be required in the interpretation of the potentials observed, especially those noted while oxygen is being removed from the system by deaeration. As far as is known there are not present in bouillon electromotively active sub-

stances which stand in equilibrium with molecular oxygen according to the equation:

$$\text{Oxidized form} = O_2 + \text{reduced form.}$$

Conant[4] found that oxyhemoglobin is not electromotively active; the potentials of the system methemoglobin-hemoglobin are affected by deaeration because of the removal of hemoglobin from participation in the electrochemical equilibrium by its transformation into the inactive oxyhemoglobin. Degradation products of hemoglobin may well be present in bouillon, and may provide a dissociable mechanism through which removal of oxygen affects the potentials.

We may question the existence of a suitable system to record more positive potentials than those observed, for although the addition of H_2O_2 in suitable amount raises the potential to a value exceeding +0.400 volt, there may be introduced thereby a new electromotively active system.

Since the removal of oxygen by deaeration or by combination with some constituent of the medium discloses a reduction intensity in bouillon corresponding to −0.060 volt, it is probable that any other process by which oxygen is withdrawn as reagent would result, in the absence of added factors of oxidation or reduction, in the attainment of the same potential level. Active bacterial respiration is such a process for the withdrawal of oxygen. But since *sterile* bouillon may attain the potential level indicated, the development of this degree of reduction intensity in bacterial cultures cannot be attributed to reductive processes directly dependent upon the activity of living cells. The potentials attained during growth of *B. typhosus* in sugar-free bouillon and their interpretation in the light of this conclusion will form the subject of a second paper.

The time potential curves of sterile bouillon, which show the course of potentials during deaeration with nitrogen are very similar to those obtained by Kenny[6] in a study of the oxidation-reduction equilibrium of tomato and cabbage juice. The equilibrium value of tomato juice calculated for pH 7.6 corresponds closely with that observed for bouillon. It is probable therefore that the potential relations described for bouillon are not peculiar to that system, but involve factors which are concerned in the behavior of biological solutions in general.

SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

IV. Penetration of Trimethyl Thionin into Nitella and Valonia from Methylene Blue.

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(Accepted for publication, May 15, 1928.)

I.

INTRODUCTION.

Methylene blue has been generally regarded as a vital stain, but recent experiments[1,2] have shown that it is not necessarily methylene blue that stains living cells. Using very large cells (*Valonia macrophysa*) yielding enough sap for spectrophotometric analysis, it was found that the dye in the sap of the vacuole is mostly, if not entirely, azure B, which is present as an impurity[3] in the external solution of methylene blue. Methylene blue was not found in the sap even in reduced form.

In order to discover whether these results have general validity it became necessary to perform similar experiments on the fresh water plant *Nitella*[4] and the marine alga *Valonia;* the results of this comparison are given in the present paper.

[1] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425.

[2] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 927.

[3] The following investigators have stated the presence of methylene azures in methylene blue. Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. MacNeal, W. J., *J. Infect. Dis.*, 1925, xxxvi, 538. Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358.

[4] Preliminary reports have been published (*cf.* foot-notes 1 and 2, and Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927–28, xxv, 563).

II.

Methods.

1. Conditions of the Experiments.—The experiments were made with *Nitella flexilis* and *Valonia macrophysa*. The dye used for *Nitella* was dissolved in phosphate or borate buffer solutions at pH 5.5 or 9.2 (made up according to the standards given by Clark,[5] and diluted ten times). The pH values of the buffer solutions were checked by means of the hydrogen electrode. The dye used for *Valonia* was dissolved in sea water at pH 9.5 (0.8 cc. of 0.2 N NaOH added to 100 cc. of Bermuda sea water). The pH value was determined as previously described.[6] The concentration of dye in the sap was determined[7] by matching the color of the tube containing the sap with that of the tube containing a known concentration of dye. The experiments were carried out at 25 ± 0.5°C. Care was taken not to expose cells to direct sunlight. The solutions were changed every hour.

2. Criteria for the Condition of the Cells.—Irreversible injury is very often followed by collapse of the cell and disintegration of the chlorophyll bodies, but the loss of turgidity if not too far advanced is often found to be reversible. Reversible injury cannot be satisfactorily determined. Turgidity seems to be the best criterion, but the outcome will vary with the judgment and experience of the observer. If we employ this criterion and find that cells die in the solutions within 24 hours, are we justified in thinking that the cells are not injured when placed in such solutions for only 1 hour? Here again the outcome will vary with the personal judgment of the experimenter, though this is one of the methods used for controlling the experiments.

The least reliable method of detecting injury is to compare the mortality of the test cells (which have been transferred after the experiment from the dye solution to the normal medium) with the mortality of the control cells. If such cells begin to die very shortly after they are transferred we may conclude that in all probability severe irreversible injury occurred before the cells were removed from the dye solution, but if after a longer period they begin to die more rapidly than the control cells we are in no position to decide whether they were injured in the original solution or died as a result of the subsequent toxic action of the dye which had not been washed away (from the cell surface or the interior). In case these cells after transference are found to live just as well as the control cells, we are in no position to say that the cells had not been reversibly injured before transference since they may very well have been injured and recovered after transference.

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[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, pp. 81 and 83.

[6] *Cf.* foot-note 2.

[7] Test-tubes were employed for *Valonia* and capillary tubes for *Nitella*.

On the basis of the first two criteria the results presented in this paper involve no injury, unless otherwise stated.

3. Method of Collecting the Sap for Spectrophotometric Analysis.—Owing to the fact that the volume of sap collected from the vacuole of each cell of *Nitella* is very small there is danger of contamination of the sap from the stained cell wall at the time of extraction of the sap from the vacuole. Contamination may occur whether the sap is extracted by puncturing the cell wall with a capillary tube and drawing the sap into it or by cutting one end of the cell and allowing the sap to flow out into a glass container. To avoid contamination the cells were placed with only about ¼ of their length in the solution, as will be described later. About six cells were placed in each dish and were removed after a few minutes. It took about 1 hour to extract sufficient sap (1 cc.) for spectrophotometric analysis.

With *Valonia* the cells were completely immersed in the dye solution. After they were taken out of the solution, the superfluous dye was removed as much as possible in the following manner. The cells were wiped with cheese cloth dampened with the sea water in which they are normally kept, dipped in the sea water, wiped again with the dampened cloth, and finally wiped with a dry cloth. Even after all this washing and wiping, the dye still comes out onto the cloth when cells are wiped. The sap was extracted from the vacuole by puncturing the cell wall with a sharp capillary end of a glass tube into which the sap was drawn up. It took about 10 minutes to extract sufficient sap for analysis. Medium sized cells were chosen (the sap from each cell would have a volume of about 0.4 cc.). Unless otherwise stated the amount of dye penetrating from solutions at pH 9.5 into the vacuoles of such cells was sufficient to make the error resulting from contamination of the sap from cell wall at the time of extraction relatively negligible.[8]

The vial containing the extracted sap of *Nitella* or *Valonia* was shaken very gently several times to mix the contents. The sap was drawn up into a pipette and placed in the cell for immediate spectrophotometric analysis unless otherwise stated. The analysis itself took about ½ hour. Measurements at critical wave-lengths (between 645 mμ to 670 mμ) were determined twice, once before the regular series of measurements were made and a second time when the usual series were measured from 550 mμ to 690 mμ (proceeding from the lower to the higher wave-lengths). In no case were the readings found to differ which showed that the light had not affected the dye during the measurement.

4. Accuracy of the Measurements.—The concentration of the dye and the thickness of the layer of solution were adjusted as much as possible to ensure accurate measurements. The thickness of the layers employed for each curve is described in Table I.

The solvent for dye employed was (1) either the sap of *Nitella* containing about 0.11 M halides (mostly potassium chloride) at pH 5.5, or (2) diluted buffer solution (described in the text), or (3) sap of *Valonia* containing 0.6 M halides (mostly potassium chloride) at pH 5.8, or (4) sea water (0.58 M halides).

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[8] *Cf.* foot-note 2 (p. 929).

As already stated in a previous publication[2] the shape of the absorption curve differs with varying concentrations of dye and of salt present in the solvent. An increase in the concentration of either one tends to accentuate the secondary absorption maximum and obscure the primary, without disturbing actually the position of these maxima in relation to the wave-lengths. For example, irrespective of the concentration the primary absorption maximum of trimethyl thionin or azure B is at about 650 mμ while the secondary is at about 600 mμ, though with an increase in concentration the primary absorption maximum tends to become less distinct, and the secondary more accentuated.

For identification of dyes the primary absorption maximum is more important than the secondary and if dilution of the dye accentuated the former, we might at first sight suppose that the greater the dilution the greater the accuracy *ad infinitum*. But this is not found to be the case. Beyond a certain dilution the measurement becomes very inaccurate on account of the distortion of the spectra.

TABLE I.

Showing the Thickness of Layers of Solution in Centimeters Used for Spectrophotometric Analysis. Nature of the Solvents is Described in the Text.

Figures	Symbols	Thickness of layer
		cm.
1	All	1.0
2	×, ▲, and ●	1.0
	□	0.6
	○	0.2
3	▲	1.0
	●, ■, ×, and ○	0.3
4	All	1.0

The average eye is relatively insensitive in the spectral region of the absorption maxima of methylene blue and azure B or trimethyl thionin (between 645 and 670 mμ), and particular care was taken to avoid the distortion of spectra which might have occurred through minor errors in measurements carried out on solutions which were too lightly or too strongly colored for optimum results. The collaboration of experienced technicians in the spectrophotometric analysis of dyes was obtained to insure reliable results.

5. Acknowledgments.—Unless otherwise stated, the measurements were made by the use of the Hilger wave-length spectrometer with a Nutting photometer or Bausch and Lomb (improved model) spectro-

photometer by W. C. Holmes[9] of the Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C. I wish to express my deep appreciation to Mr. Holmes for his collaboration and to the Color Laboratory for its hospitality.

At the request of Mr. W. C. Holmes, a few measurements were repeated with a König-Martens spectrophotometer by Dr. K. S. Gibson of the Bureau of Standards, for whose collaboration as well as for the hospitality of the Bureau of Standards I wish to express my gratitude.

I also desire to thank the Marine Biological Laboratory at Woods Hole for the use of facilities during last summer.

I am greatly indebted to Mr. Holmes, Dr. B. Cohen, and to Sergeant French for samples of dyes. The samples are designated throughout the paper by the name of the donor placed in parentheses. The samples furnished by Dr. Cohen were Samples G and F of Dr. Mansfield Clark.[10]

III.

Spectrophotometric Analysis.

A. Experiments on Nitella flexilis.

(*a*) *Collected in New York.*—The experimental procedure was as follows: It was first of all necessary to determine whether the dye was stable in solution and to find out what penetrates the cell. This led to an investigation of errors due to contamination of the sap during extraction in proportion to the rate of penetration which in turn led to a study of the effects of injury. Consideration was then given to the important question whether azure B penetrates as such or is produced from methylene blue after the latter has entered the cell.

The results tend to indicate that azure B penetrates very much more rapidly than methylene blue.

[9] Cooperating expert in the field of spectroscopy of dyes for International Critical Constants.

[10] Clark, W. M., Cohen, B., and Gibbs, H. D., Hygienic Laboratory Bulletin No. 151, 1928, p. 174.

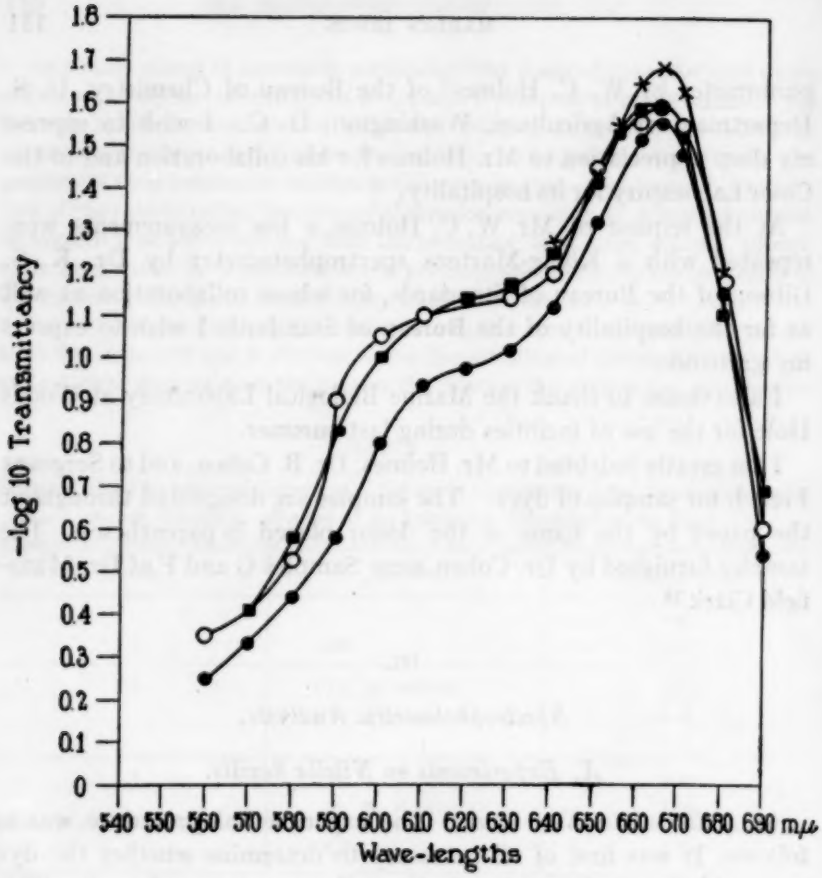

FIG. 1. Absorption curves showing that the sample of methylene blue in solution remains unchanged and that there may be contamination of the sap from cell wall at the time of extraction of sap from the vacuole of *Nitella flexilis* (New York) placed in methylene blue solution (French). Analysis made about 20 hours after extracting the sap or dissolving the sample. Symbol ● represents methylene blue dissolved in borate buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye extracted by distilled water from the cell wall of uninjured cells stained in the methylene blue solution at pH 5.5 before the dye has penetrated into the vacuole. Symbol ■ represents the dye extracted from vacuoles of cells immersed completely in methylene blue solution at pH 9.2 where contamination may play a predominant rôle. Symbol ○ represents the dye extracted from vacuoles of cells placed in methylene blue solution at pH 5.5, where contamination occurred without any penetration. The heights of these curves have no significance other than attempts to select a condition as favorable for accurate measurement as possible under these circumstances.

1. Stability of the dye solution: Methylene blue solution (French) either at pH 9.2 or at pH 5.5 gives an absorption curve characteristic of methylene blue with absorption maximum at 664 mμ (Fig. 1, symbol •) whether it is examined immediately after dissolving or 3 hours later.

2. Nature of the dye entering the vacuole when error from contamination was not avoided: Analysis was made of the sap extracted from the cells which had been completely immersed for a few minutes in 0.01 per cent methylene blue solution (French) at pH 9.2. The dye in the sap gave an absorption curve characteristic of a mixture of methylene blue and azure B with a primary absorption maximum at about 661 mμ (Fig. 1, symbol ■). Owing, however, to a severe contamination of the sap from the stained cell wall at the time of expressing the sap this result cannot be taken as conclusive evidence that the methylene blue present in the sap is a result of penetration rather than of contamination. Let us now consider the question of contamination.

3. Contamination of the sap during the process of extraction:

(*a*) In the solution just mentioned a very deep staining of the cell wall occurs before penetration of dye into the vacuole takes place. Dye extracted from the cell wall of such cells consisted chiefly of methylene blue with a primary absorption maximum at 664 mμ (Fig. 1, symbol ×). This is important since the cell wall can give off this dye to the sap during the process of extraction.

Cells were placed in the 0.01 per cent methylene blue solution (French) at pH 5.5 or at pH 9.2 and were removed after the cell wall had become heavily stained but before any penetration into the vacuole took place. Such cells were removed from the dye solution, wiped, and were then placed in a small volume of distilled water until sufficient dye for analysis was extracted from the cell wall. Several extractions were analyzed and they were found to give the same results. Similar results were obtained when the extraction from the cell wall was made after the sap and the protoplasm had been removed.

(*b*) Sap known to owe most of its color to contamination at the time of extraction showed an absorption curve characteristic of methylene blue with an absorption maximum at 664 mμ (Fig. 1, symbol ○).

Cells were placed in a solution of methylene blue (French) at pH 5.5 until the cell wall was deeply stained. The end of the cell was cut and a drop of sap was

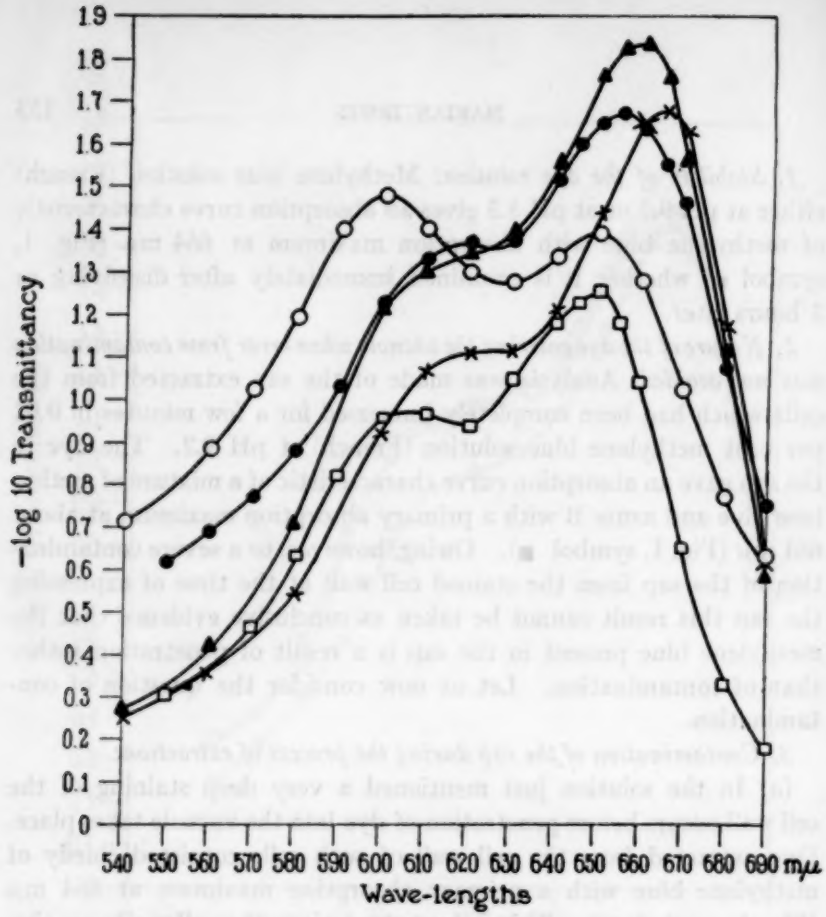

FIG. 2. Absorption curves showing the nature of the dye collected from the vacuole of *Nitella* (New York) placed in solutions of methylene blue (French) or azure B (Holmes), analysis being made about 20 hours after extracting or dissolving the sample. Symbol ● represents the dye collected from the vacuole of living cells after several minutes exposure to methylene blue solution at pH 9.2, where contamination was eliminated by allowing part of the cell to project out of the solution as described in the text. Symbol × represents methylene blue (French), at about the same concentration as the dye represented by symbol ●, dissolved in freshly extracted sap and analyzed after a period equal to the duration of penetration experiment and subsequent measurement. Symbol ▲ represents the dye collected in the vacuole of cells which have lost some turgidity as the result of injury during exposure to methylene blue solution at pH 9.2 where contamination was likewise eliminated. The dye in the sap was diluted with freshly extracted sap containing no dye since it was too concentrated for measurement. Symbol □ represents the pure sample of azure B (Holmes) dissolved in borate buffer solution at pH 9.2. Symbol ○ represents the dye collected from the vacuole of living cells after a few minutes exposure to the azure B solution at pH 9.2, where contamination was eliminated. The heights of these curves have no significance other than an attempt to adjust the concentration and thickness of the layer of solution so as to obtain as accurate a measurement as possible under these circumstances.

allowed to fall onto a glass slide. The drop of sap appeared colorless except for a very dark blue spot in the center which was caused by the dye diffusing from the cell wall contaminating the sap. The contamination occurred when the hanging drop of sap came in contact with the stained cell wall before it separated from the cut end and flowed out upon the surface of the glass slide.

These results show that the error may arise from contamination of the sap from the stained cell wall at the time of extraction. It was therefore necessary to avoid this contamination.

4. Nature of the dye entering the vacuole of uninjured cells when contamination was eliminated: To avoid contamination only about $\frac{3}{4}$ of the length of the cell was immersed in the methylene blue solution (French) and the remaining $\frac{1}{4}$ was allowed to rest on the edge of the dish. The latter was kept moist by wet absorbent cotton which, however, was separated from the dye solution by a short segment of the cell. Cells were thus placed in 0.01 per cent dye at pH 9.2 for a few minutes. The dye in the sap of such cells was found to give an absorption curve characteristic of chiefly azure B and a smaller percentage of methylene blue with a primary absorption maximum at 655 mμ (Fig. 2, symbol ●). When the sap thus extracted was exposed to the air in an alkaline solution the color of the dye in the sap did not deepen, thus showing that there was no methylene blue in reduced form in the sap.

5. Nature of the dye penetrating the vacuole of injured cells: Since reversible injury is difficult to detect, we may inquire whether the azure B found in the vacuole as described under (4) is a demethylation product of methylene blue as associated with injury. If this were the case, the more injury the more azure B would be formed. To test this point experiments were carried out with cells which began to lose their turgidity during the experiment. It was found that methylene blue penetrated the vacuole more rapidly when cells began to lose their turgidity, which is shown by the fact that the dye from the vacuole then has a primary absorption maximum at 659 mμ (Fig. 2, symbol ▲).

Obviously azure B penetrates much more rapidly than methylene blue unless injury has occurred. But it is possible that this penetration is only apparent and that after all it is methylene blue which penetrates and is transformed to azure B after it has entered. We must now consider this question.

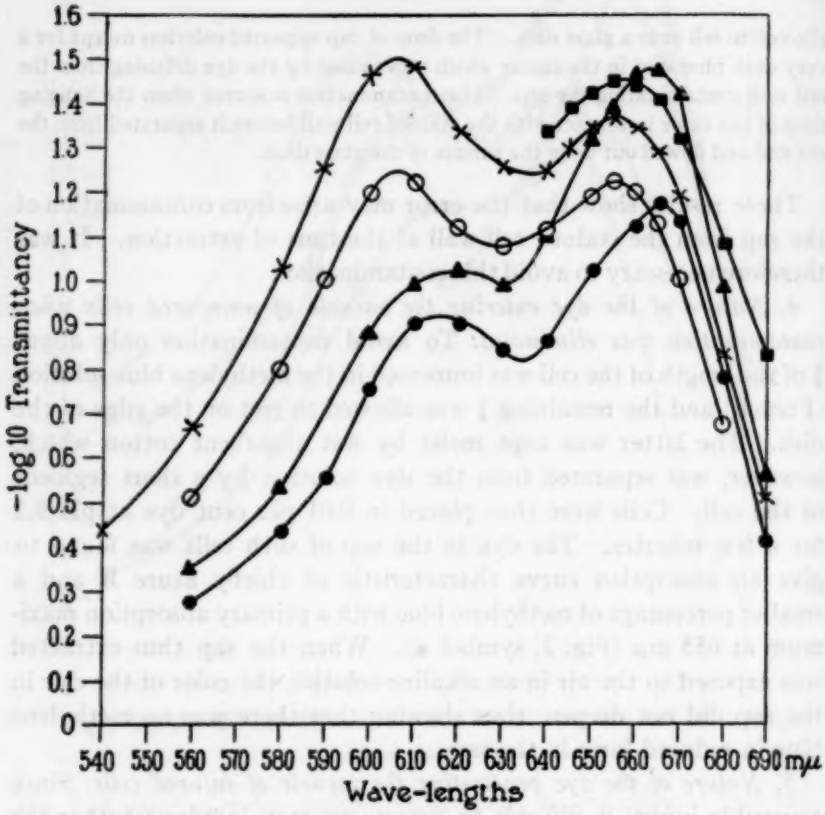

FIG. 3. Absorption curves showing the results obtained with *Nitella* (collected in Massachusetts) proving that the results are identical whether analysis is made immediately or 20 hours after extraction. Symbol ● represents the sample (Merck's medicinal) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ■ represents the dye collected from the vacuole of living cells placed in methylene blue (Merck's medicinal) at pH 9.2, when analysis was made immediately after extraction. Symbol ▲ represents methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 and analyzed 4 hours later. Symbol × represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2, and analyzed immediately after extraction. Symbol ○ represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2 and analyzed 20 hours after extraction. The heights of these curves have no significance other than an attempt to make measurement in the region where accuracy is possible under these circumstances. Contamination was avoided in all cases.

6. Is the azure B in the sap produced from methylene blue which has entered the cell? To throw some light upon this question methylene blue (French) was dissolved in freshly extracted sap (at about the same concentration as that found in the vacuole in penetration experiments) and allowed to stand for about the same length of time as was the case with the penetration experiment followed by the measurement. The dye in the sap was found to give an absorption curve characteristic of methylene blue with a primary absorption maximum at 664 mμ (Fig. 2, symbol ×). This indicates that the sap is not able by itself to change methylene blue to azure B to any noticeable extent during the time of experiment.

(*b*) *Collected in Massachusetts.*—Owing to the fact that the extraction of sap from the vacuole of cells of *Nitella* required the cooperation of several technicians it was necessary in the experiments just described to extract the sap in New York City while the measurements were made by Mr. Holmes in Washington. Although these measurements were made about 20 hours after extraction of the sap, the validity of these analyses is shown by the fact that later, when it was possible to make in Woods Hole extractions followed immediately by measurements the dye in the sap (extracted from cells collected in Massachusetts, care being taken to avoid contamination) gave the same absorption curve as before whether it was measured immediately after extraction or 20 hours later. The primary absorption maximum was at 655 mμ (Fig. 3, symbols × and ○). This primary absorption maximum closely resembles that obtained with New York *Nitella* (Fig. 2, symbol ●).

The same result was obtained when the experiments were repeated with methylene blue (Merck's medicinal) (Fig. 3, symbols ●, ■, and ▲).

Experiments with pure azure B. Although the sap has been shown to be incapable of demethylating methylene blue to azure B is the protoplasm able to do so? Since Holmes[11] has found that demethylation of azure B to azure A takes place more readily than demethylation of methylene blue to azure B, if we find azure B to penetrate the vacuole without demethylation, from pure azure B solution, we may be justi-

[11] Holmes. W. C., to be published later.

fied in concluding that it is unlikely that the protoplasm is capable of changing methylene blue to azure B. Dye from cells placed in azure B solution (Fig. 2, symbol □) gives an absorption curve characteristic of azure B with a primary absorption maximum at 650 mμ (Fig. 2, symbol ○), thus showing that in all probability neither the protoplasm nor the sap is capable of demethylating azure B (contamination was avoided).

B. Experiments on Valonia macrophysa.

Dye from uninjured cells left for about 1½ hours in methylene blue (French) at pH 9.5 gave an absorption curve characteristic of azure B with an absorption maximum at 650 mμ (Fig. 4, symbol ●), while the external solution consisted chiefly of methylene blue with an absorption maximum at about 664 mμ (Fig. 4, symbol □).

If the conclusion is correct that the presence of azure B in the vacuole of cells placed in methylene blue solution is due to the more rapid penetration of azure B from the outside solution (which contains azure B as impurity) we should expect an increase in the rate of penetration on the addition of more azure B to methylene blue. This was found to be the case experimentally. Enough dye for analysis collected in half an hour in the vacuoles of cells (at pH 9.5) in 0.04 per cent methylene blue solution (Merck's medicinal) to which about 25 per cent of the same concentration of azure B was added, whereas during the same period not sufficient dye for analysis penetrated from the same sample of methylene blue solution to which no addition of azure B was made. Spectrophotometric analysis of the first solution showed a mixture of methylene blue and azure B with a primary absorption maximum at 660 mμ (Fig. 4, symbol ○) while the dye in the vacuole was chiefly azure B with a primary absorption maximum at 650 mμ (Fig. 4, symbol ▲).

When cells are placed in pure azure B solution (Holmes) with a primary absorption maximum at 650 mμ sufficient dye for analysis enters in a very few minutes and this dye is found to be azure B with a primary absorption maximum at 650 mμ (Fig. 4, symbol △) which indicates that neither the protoplasm nor the sap is capable of changing azure B.

All these measurements were made immediately after extraction of the sap. When some of the measurements in connection with *Valonia* were repeated by Dr. Gibson with a König-Martens spectrophotometer the results confirmed those previously obtained. Whether Samples F and G of Clark, or the sample of French is used, the dye which

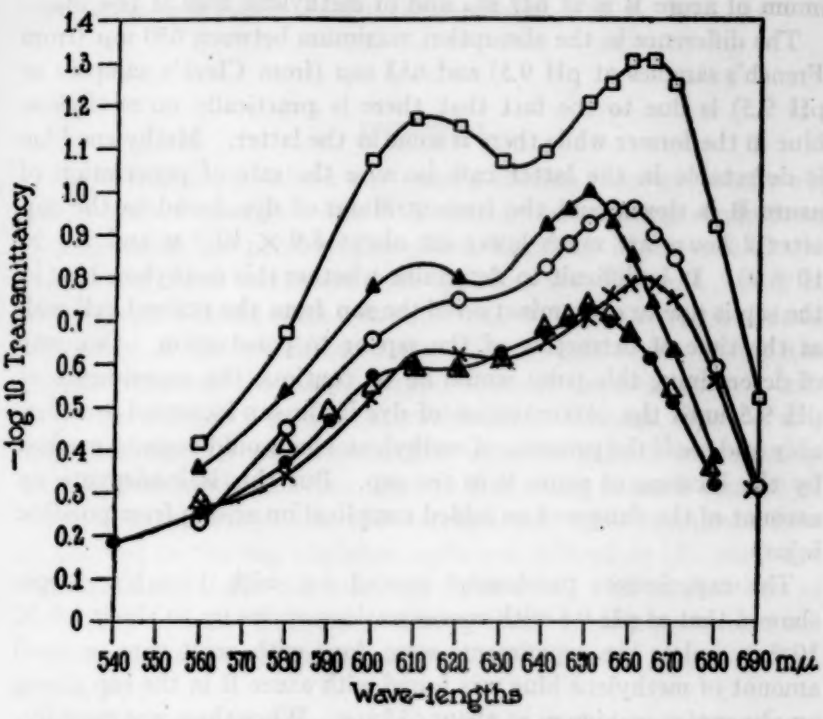

FIG. 4. Absorption curves showing the penetration of dye into *Valonia* from a mixture of azure B and methylene blue in comparison with the dye from methylene blue solution containing a trace of azure B as impurity. Symbol □ represents methylene blue (French) dissolved in sea water at pH 9.5 and analyzed 4 hours later. Symbol ● represents the dye which has penetrated from this solution in 1½ hours when analysis was made immediately after extraction. Symbol × represents methylene blue (French) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ○ represents a mixture of about 75 per cent of methylene blue and 25 per cent of azure B at pH 9.2. Symbol ▲ represents the dye which has penetrated from this solution in ½ hour when analysis was made immediately after extraction. Symbol △ represents the dye which has penetrated from pure azure B solution in less than ½ hour.

collects in the vacuoles of uninjured cells of *Valonia macrophysa* placed in methylene blue solution at pH 9.5 gives an absorption curve characteristic of dye chiefly consisting of azure B. With the sample of French, this absorption maximum is at 649 mμ while with Clark's samples it is at about 653 mμ.[12] The absorption maximum of azure B is at 647 mμ and of methylene blue at 664 mμ.

The difference in the absorption maximum between 650 mμ (from French's samples at pH 9.5) and 653 mμ (from Clark's samples at pH 9.5) is due to the fact that there is practically no methylene blue in the former while there is some in the latter. Methylene blue is detectable in the latter case because the rate of penetration of azure B is slower and the concentrations of dye found in the sap after 2 hours are much lower (at about 5.9×10^{-6} M and 7.9×10^{-6} M). It is difficult to determine whether this methylene blue in the sap is due to contamination of the sap from the stained cell wall at the time of extraction of the sap or to penetration. One way of determining this point would be to continue the experiments at pH 9.5 until the concentration of dye in the sap increased considerably and see if the presence of methylene blue would become masked by the increase of azure B in the sap. But this is inadequate on account of the danger of an added complication arising from possible injury.

The experiments previously[2] carried out with French's sample showed that at pH 9.5 with concentrations of dye up to about 1.6×10^{-5} M, unless the experiments were done with great care, a small amount of methylene blue was found with azure B in the sap giving an absorption maximum at about 653 mμ. When there was more dye in the sap the methylene blue was masked by azure B, thereby giving an absorption maximum at 650 mμ which was identical with that of pure azure B. This indicates that at lower concentrations of dye in the sap there is a considerable possibility of error arising from contamination.

[12] Experiments were continued up to 3½ hours when one reading gave an absorption maximum at 653 mμ and the other at 656 mμ. At such a slow penetration it is impossible to determine whether the presence of methylene blue (together with azure B) in the sap is due to very slight injury, contamination, or very slow penetration (much slower than the penetration of azure B). The results of such experiments must therefore be considered doubtful.

These results do not disagree with the theory presented in the text since they show that at higher external pH value the dye in the vacuole of uninjured cells consists chiefly of azure B while the external solution contains chiefly methylene blue with a trace of azure B as impurity. This indicates that azure B in form of free base possibly penetrates more rapidly than methylene blue.

IV.

DISCUSSION.

These results on *Valonia* and *Nitella* confirm those previously obtained with *Valonia*.[1,2] The fact that by eliminating errors from contamination the primary absorption maximum in the case of dye penetrating from methylene blue solution at about pH 9.5 is at 650 mμ with *Valonia* while it is at 655 mμ with *Nitella* may be due to differences in the condition of the cells brought about during the experiments or to the normal compositions of the two types of cells or to the difference in the external media. Though there is this difference, the fact still remains that at about pH 9.5 both take up azure B from methylene blue solution containing a small amount of azure B as impurity. At this external pH value methylene blue is detected in the sap (1) when cells are injured or (2) when the contamination of the sap from the stained cell wall at the time of extraction occurs. The errors arising from these two sources are frequent so that experiments must be carried out with a great deal of care.

Has this azure B actually penetrated as such from the external solution or has methylene blue entered and become demethylated to form azure B? Holmes[13,14] has shown that a progressive demethyla-

[13] Since Holmes has found that demethylation of methylene blue to azure B at pH 11 does not occur instantly but only after standing for a long period this increase in the rate of penetration of azure B with a rise in the external pH value to pH 10.9 where at maximum the solution has stood for only 1 hour is not due to the increase in the concentration of azure B resulting from demethylation of methylene blue in presence of greater akalinity.

[14] Transformation of methylene blue to methylene azure in presence of alkalinity has been found by others. Bernthsen, A., *Ann. Chem.*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxviii, 4.

tion of methylene blue to azure B and then to azure A does not take place readily at a pH value below 10. Since the pH value of the sap is at about pH 5.8 and since the work of others[15] indicates the pH value of the protoplasm in general to be considerably below 10, we may conclude that methylene blue cannot be demethylated by the protoplasm nor by the sap at their normal pH values unless they contain a substance capable of bringing about demethylation at a pH value below 10. Such substances must be absent from the sap for freshly extracted sap is incapable of demethylating methylene blue (at the concentration of the blue dye actually penetrating the vacuole) even during a period longer than that required for the penetration and its subsequent measurement in my experiments.

An additional reason for doubting that the protoplasm and the sap of these cells can demethylate methylene blue is that azure B is found to penetrate from pure azure B solution into the vacuole as such without undergoing demethylation to azure A. Since Holmes[11] has found that demethylation of methylene blue to azure B is brought about less readily than that of azure B to azure A, it is unlikely that the system which is incapable[16,17] of changing azure B could transform methylene blue.

The azure B is not formed from methylene blue as a result of the change in the protoplasm or in the sap caused by an injury, because so long as cells are not injured azure B collects in the vacuole while as soon as it becomes injured methylene blue begins to penetrate freely.

The spectrophotometric measurement of the dye in the vacuolar sap of *Valonia macrophysa* has been recently repeated by M. M. Brooks,[18] who concludes that the azure B present in the vacuole in my experiments is due to the transformation of methylene blue by the

[15] A review of work on the pH values of the protoplasm is given by Chambers, R., *J. Gen. Physiol.*, 1926–27, x, 739.

[16] This statement, however, must be made with reservation, since it has been found that in dogs the methyl group in position seven of the xanthine ring is most readily removed while in rabbits the opposite is the case (Krüger, M., and Schmidt, J., *Ber. chem. Ges.*, 1899, xxxii, 2677; *Z. physiol. Chem.*, 1902, xxxvi, 1).

[17] When methylene blue is injected intravenously azure B is found in the urine and feces (Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358).

[18] Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90.

sap after extraction. She bases this conclusion on the assumption that my measurements were made after a lapse of time sufficient for the transportation of the extracted sap from New York to Washington. In view of the fact that my measurements, even when completed within 1 hour after extraction, gave absorption curves characteristic of azure B there is no basis for her conclusion.

If we suppose that azure B penetrates as such, diffusing through the protoplasm from an external solution containing mostly methylene blue, with only a small percentage of azure B, we may conclude that azure B penetrates the vacuole much more rapidly than methylene blue. This conclusion is supported by experiments showing that azure B collects in the vacuole much more rapidly from pure azure B than from methylene blue solution. The behavior of azure B is similar to that of many other basic dyes which are capable of forming free base and salt, since it is found to penetrate the vacuole much more readily as free base than as salt (the higher the pH value of the external solution the more rapid is the rate of penetration). This increases with rising pH value of the external solution for both azure B and methylene blue.[19,20,21]

These results are in agreement with the theory[22] that the rate of entrance of the dye is higher the more rapidly it enters the non-aqueous layers of the protoplasm and passes from them into the

[19] The following investigators have found an increase in the rate of penetration of dye with an increase in the external pH value of methylene blue solution. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350.

[20] My statement showing that the blue dye penetrated the vacuole of living cells from methylene blue solution more rapidly at pH 9.5 than at pH 5.5 (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, foot-note 15, p. 572; 1926–27, x, 927) was contrary to the first conclusion drawn by Brooks, M. M. (*Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 265; *Am. J. Physiol.*, 1926, lxxvi, 360), but my conclusion was confirmed by her in her later publication (Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90).

[21] Baudisch and Unna have found that methylene azure enters chloroform and appears red (see foot-note 14). Kehrmann (14) has stated that methylene azure enters ether, chloroform, and benzene in form of a base and not in form of a salt.

[22] Irwin, M., *J. Gen. Physiol.*, 1927–28, xi, 112; *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127.

vacuolar sap. The entrance of the dye therefore depends on the concentration gradient and on the partition coefficient between the non-aqueous layers of the protoplasm and the aqueous phases.

In the present case the cell behaves as if the rate is controlled by only three phases (1) external solution, (2) one non-aqueous layer, and (3) aqueous sap in the vacuole. Since this will form the subject of the next paper it will not be discussed at length here.

On basis of this theory we may expect a very slow penetration of both azure B (in form of salt) and methylene blue, which might be detected if the dye penetrating the vacuole from methylene blue solution at pH 5.5 could be measured, but unfortunately the penetration is too slow for accurate analysis.

If methylene blue penetrates, its presence might be detected if there were little or no penetration of azure B. This might be ensured by placing cells in a sample of methylene blue exceptionally free from azure B but in this case the penetration of the blue dye is so slow that we are unable to obtain data and errors may result from injury or contamination.

Although the experiments show that unless cells are injured, azure B collects in the vacuole much more rapidly than methylene blue from methylene blue solution at a higher pH value, they do not tell us anything about the penetration of methylene blue into the protoplasm (which forms a layer so thin that no experiments suitable for our present purpose can be made on it directly). They merely point out the danger of drawing any conclusion as to the permeability of cells or the oxidation reduction potential of cells based on penetration of a blue dye from methylene blue solution without satisfactory determinations of the nature of the dye inside and outside the cell.

SUMMARY.

Spectrophotometric measurements show that it is chiefly the trimethyl thionin that is present in the sap extracted from the vacuoles of uninjured cells of *Nitella* or *Valonia* which have been placed in methylene blue solution at a little above pH 9. Whether these measurements were made immediately or several hours later the same results were obtained. Methylene blue is detected in the sap

(1) when the cells are injured or (2) when the contamination of the sap from the stained cell wall occurs at the time of extraction.

The sap is found to be incapable of demethylating methylene blue dissolved in it even on standing for several hours.

It is somewhat uncertain as to whether the trimethyl thionin penetrated as such from the external methylene blue solution which generally contains this dye as impurity (in too small concentration for detection by spectrophotometer but detectable by extraction with chloroform), or whether it has formed from methylene blue in the protoplasm. The evidences described in the text tend to favor the former explanation.

Theory is discussed on basis of more rapid penetration of trimethyl thionin (in form of free base) than of methylene blue, or of trimethyl thionin in form of salt.

THE DEATH WAVE IN NITELLA.

I. Applications of Like Solutions.

By W. J. V. OSTERHOUT and E. S. HARRIS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Accepted for publication, May 15, 1928.)

Experiments with chloroform have led us to predict[1] that the current of injury will be positive when the cell is in contact with concentrated solutions (such as 0.1 M KCl) and negative with dilute solutions (such as 0.001 M KCl).

The experiments on cutting here described justify this prediction. They also reveal a new feature: the rapid spread of the effects of injury,[1] a study of which may assist our understanding of the propagation of stimuli. From the point where the cell is cut a wave of some sort, which we may for convenience call the death wave, passes along the cell, setting up at each point it touches a death process which has greater speed and intensity the nearer it is to the cut.

The experiments were performed on *Nitella flexilis*, arranged as shown in Figs. 1, 4, or 6: the changes in potential difference being recorded photographically by means of a string galvanometer. The technique has been fully described in a previous paper. The average temperature was about 23°C. but as the experiments were very brief there was little variation in any one experiment.

The results remained practically the same when the pH value of the solutions varied from 4 to 9.

Let us first consider the experiments in which sap[2] or 0.05 M KCl was applied at *A* and *B* (Fig. 1) after which the cell was cut at *Z* by means of a sharp knife insulated by rubber from contact with the hand (a clean cut was made by cutting downward against the paraffin

[1] This has been referred to in certain previous publications, *cf.* (*a*) Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83; (*b*) Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673.

[2] 0.05 M KCl gives the same results as sap for such experiments as are here described.

on which the cell rested). The cut caused *A* to become more positive after which the potential difference approached zero (Fig. 2).

Our interpretation of this phenomenon may be presented by describing how it arose. Our first experiments of this sort aroused the suspi-

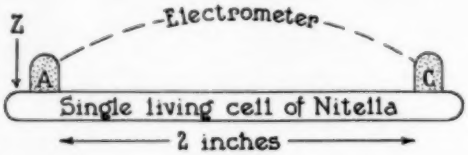

FIG. 1. Diagram to show the arrangement of the experiments (the cut is made at *Z*). Flowing junctions or pieces of cotton soaked in solution are applied at *A* and *C*. When necessary, with more concentrated solutions, cotton soaked in distilled water is applied for a short stretch between *A* and *C* but a space is left on each side of the cotton to prevent short-circuiting. In some cases the ends of the cells dip into cups holding solution.

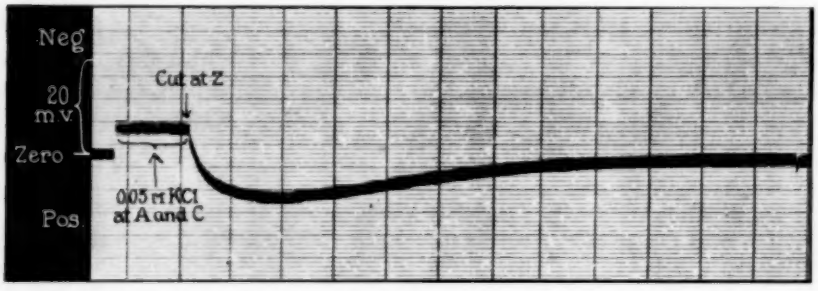

FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.05 M KCl at *A* and *C*. The curve (showing the potential difference of *A* with reference to *C*) is at first slightly negative but becomes positive when the cell is cut at *Z*, and then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

cion that all the changes produced at *A* are followed by similar ones at *C*,[3] and that the observed curve merely represents the resultant of the

[3] The microscopic appearance of *C* changed in much the same way as that of *A* and so did its response to the tests for normality described in a former paper (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 417). The hypothetical curves of *A* and *B* imagined at that time were almost the same as those we use at present.

opposing potential differences at *A* and *C*. To illustrate this we made (Fig. 3*a*) a tracing of the curve in Fig. 2 and drew (Fig. 3*b*) hypothetical *A* and *C* curves to make it evident that the difference between them would give the curve in Fig. 2. But there was no way of testing

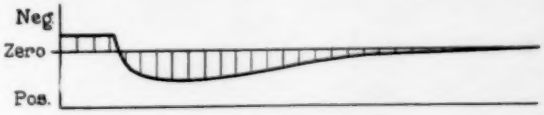

FIG. 3 *a*.

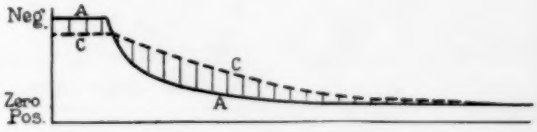

FIG. 3 *b*.

FIG. 3 *a*. Tracing of the curve in Fig. 2 with certain ordinates drawn for comparison with Fig. 3 *b*.

FIG. 3 *b*. Theoretical interpretation of Fig. 3 *a*. Fig. 3 *a* shows only the observed potential difference but Fig. 3 *b* shows that this is equal to the difference between the hypothetical curve *A* (unbroken line) and the hypothetical curve *C* (broken line): each ordinate is equal to the one directly above it in Fig. 3 *a*. *A* and *C* are the (hypothetical) "true" curves of *A* and *C*. At the start *A* is more negative than *C* which accords with the fact that the curve in Fig. 3 *a* is negative at the start (this curve shows the potential difference of *A* with respect to *C*). When the value of *A* falls to that of *C* the curve in Fig 3 *a* becomes zero; when *A* becomes positive to *C* the curve in Fig. 3 *a* becomes positive; the two curves then approach each other and the curve in Fig. *3 b* approaches zero.

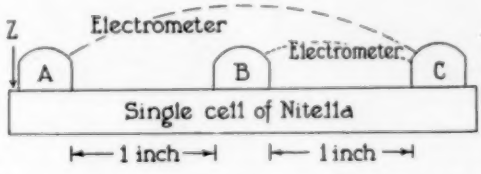

FIG. 4. Like Fig. 1 except for the addition of *B*.

this conception until it was observed that chloroform may kill *C* without immediately affecting *A* and *B*.[18]

It was found that when *C* is killed its electromotive force falls to zero and remains constant while the cut is being made at *Z* and

the resulting disturbances are recorded. This is illustrated by Fig. 5 in which the record starts after the disturbances[4] produced by chloro-

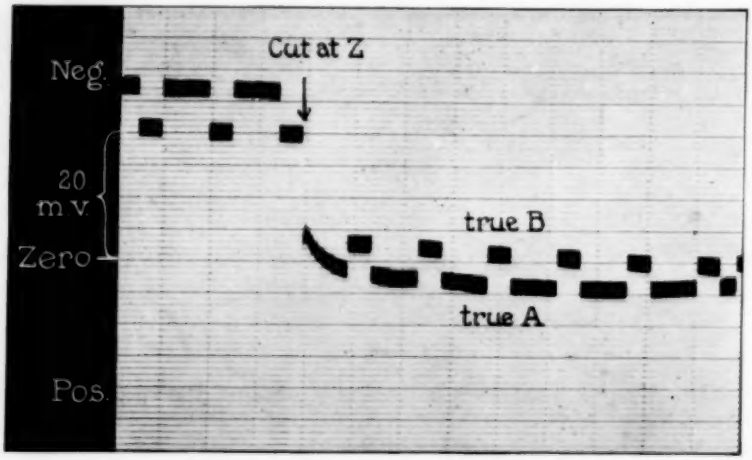

FIG. 5. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.05 M KCl at *A*, *B*, and *C*. The record begins after *C* has been killed by 0.05 M KCl saturated with chloroform so that the *A* and *B* curves have their "true" values (and hence will be called the "true" curves of *A* and *B* since the death of *C* reduces its electromotive force approximately to zero). At the start these curves are negative (*A* more so than *B*) but on cutting at *Z* they approach zero. The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

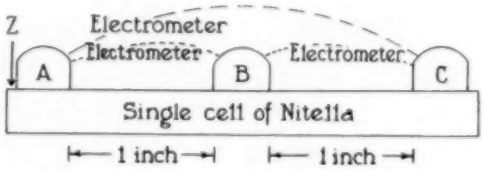

FIG. 6. Like Fig. 4 but with an additional connection between *A* and *B*.

form are over: *A* and *B* are then negative and have their "true" values (*i.e.* the values observed when the electromotive force of *C*

[4] These will not be discussed here since they have been described in a previous paper,[1b] where it is shown that the injury at *C* does not immediately spread to *A* and *B*.

is zero). After cutting they approach zero,[5] the process at *A* being a little in advance[6] of that at *B* (this is not evident in Fig. 5 but is shown by other experiments).

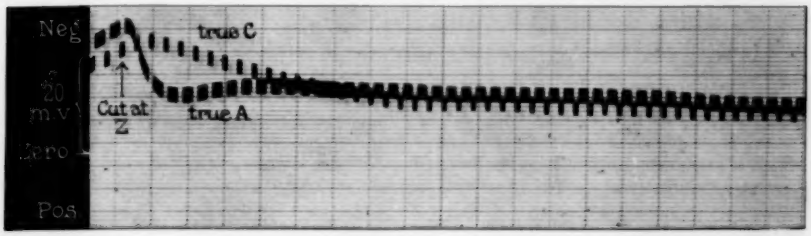

FIG. 7 *a*.

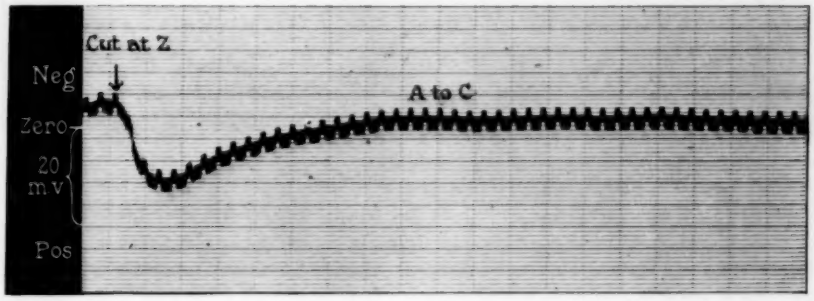

FIG. 7 *b*.

FIGS. 7 *a* and *b*. Photographic records of potential differences, the experiment being arranged as in Fig. 6 with 0.05 M KCl at *A*, *B*, and *C*. The records begin after *B* has been killed (by 0.05 M KCl saturated with chloroform) so that the curves of *A* and *C* show their "true" values (both are negative at the start, *A* being more so than *B*, but on cutting at *Z* they approach zero): these are recorded on one instrument while the curve showing the potential difference between *A* and *C* is simultaneously recorded on another as shown in Fig. 7 *b* (the slight vertical movements in this curve are due to the alternations of the rotary switch in the other instrument); the value of each point of the curve is equal to the difference between the "true" *A* and "true" *C* values. The vertical marks represent 5 second intervals. Selected as typical from 20 experiments.

[5] The curves for *A* and *B* are recorded simultaneously by means of the rotating switch previously described (Osterhout, W. J. V., and Harris E. S., *J. Gen. Physiol.*, 1927–28, xi, 417).

[6] This permits us to study the speed of propagation of the stimulus, which will be discussed in a later paper.

It would seem that leading off from A to B must be similar to leading off from A to C and that the A to C curve must resemble the A to B curve and must be approximately[7] equal to the difference between the "true" curves of A and C (and hence of A and B in Fig. 5) which would give a curve similar to that of Fig. 2. That this is really so became evident as soon as it was possible, by making simultaneous records on two instruments and using the arrangement shown in Fig. 6, to get the A to C curve together with the "true"

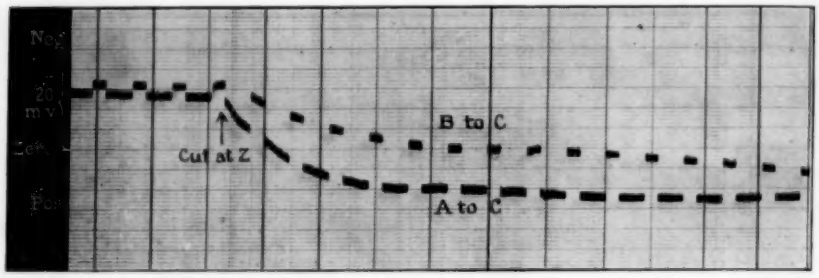

FIG. 8. Like Fig. 5 except that C is not killed. The A and B curves do not have their "true" values (as in Fig. 5) but merely show their potential difference against C. The difference between them, however, gives the same sort of curve as that shown in Fig. 2. The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

curves of A and C. Inspection of Figs. 7*a* and *b* shows that the difference between the true curves of A and C gives the observed A to C curve, *i.e.*, if we measure the vertical distance between A and C we obtain the vertical distance above or below zero of the A to C curve.

In this case we notice that the death wave which starts at Z must pass over the freshly killed protoplasm at B before it can affect C, but it can evidently do this, as shown by the behavior of C.

To ascertain whether the killing of C modifies the A and B curves we

[7] We should not expect to get the exact difference since, as shown in a former paper, we do not necessarily measure exactly the same fraction of the true E. M. F. in both cases. But this would not noticeably affect the form of the curves and might make very little quantitative difference between them.

have performed the experiment without killing *C*, the result being shown in Fig. 8. In this case the curves are negative at the start (usually they are near zero under these circumstances) but on cutting

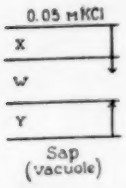

FIG. 9.

FIG. 9. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.05 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. The potential difference across the protoplasm is by convention regarded as negative since the *X* arrow is longer.

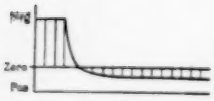

FIG. 10 *a*.

FIG. 10 *a*. Tracing of the "true" *A* curve in Fig. 5 with certain ordinates drawn for comparison with Fig. 10 *b*.

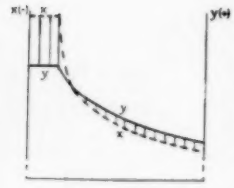

FIG. 10 *b*.

FIG. 10 *b*. Theoretical interpretation of Fig. 10 *a*. Fig. 10 *a* shows only the observed potential difference, but Fig. 10 *b* indicates that this is equal to the difference between the (hypothetical) value of *x* (broken line) with negative sign (as shown by the scale of ordinates at the left) and the (hypothetical) value of *y* (unbroken line) with positive sign (as shown by the scale of ordinates on the right): each ordinate is equal to the one directly above it in Fig. 10 *a*.

Since the potential difference across the protoplasm in contact with 0.05 M KCl is negative the *x* ordinate is made longer at the start (since *x* is negative by convention). When *x* falls and becomes equal to *y* the curve in Fig. 10 *a* falls to zero: as *x* continues to fall and becomes less than *y* the curve in Fig. 10 *a* becomes positive; when the two curves approach each other the curve in Fig. 10 *a* approaches zero.

Since we do not know the absolute value of the ordinates but only the value of the difference between them (*i.e.* the value of the shaded area) the scales of ordinates are interrupted toward the base by a dotted line to signify that below this point the ordinate extends for an indefinite distance. It should be noted that when the *x* and *y* curves come together the observed potential difference (such as is given in Fig. 10 *a*) is zero but this zero has no relation to the absolute zero of the present figure.

they become positive and then approach zero (in the latter part of the record which is not shown here). The difference between the *A* and

and B curves would evidently give a curve similar to that in Fig. 2. This is to be expected since if we designate the "true" values at A, B, and C by a, b, and c we may write as an approximation

$$\text{Observed } A \text{ to } C \text{ curve} = a - c$$
$$\text{Observed } B \text{ to } C \text{ curve} = b - c$$

Taking the difference between these two curves we have

$$\text{Observed } A \text{ to } B \text{ curve} = (a-c) - (b-c) = a - b$$

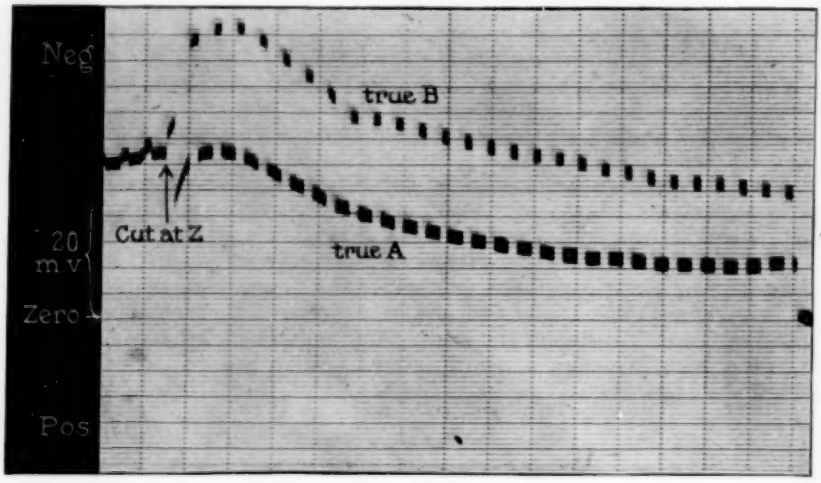

FIG. 11. Photographic record of potential differences (the experiment being arranged as in Fig. 4 with 0.05 M KCl at A, B, and C). The record begins after C has been killed (with 0.05 M KCl saturated with chloroform) so that the curves for A and B show their "true" values and are in consequence negative (their values are so nearly equal that they almost coincide). On cutting at Z, A becomes more positive, then more negative, and then approaches zero (resembling an effect often found with 0.1 M KCl): B becomes more negative and then approaches zero (resembling an effect commonly produced by 0.05 M KCl plus chloroform). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

In other words we get the observed A to B curve by taking the difference between the "true" curves of A and B or between the observed curves of A to C and B to C.

The experiments indicate that the killing of *C* does not modify the *A* and *B* curves if the cut is made soon enough after the death of *C*.

Our next step is to try to interpret the "true" curves shown in Fig. 5. These resemble in many cases the "true" curves produced by applying chloroform directly to *A* and *B* (instead of producing injury at *A* and *B* by cutting at *Z*). Such curves have been explained[1b] on the ground that the protoplasm consists of an outer layer, *X*, and an inner layer, *Y* (both of which are probably non-aqueous), with an aqueous layer, *W*, between them and that the layer in contact with the more

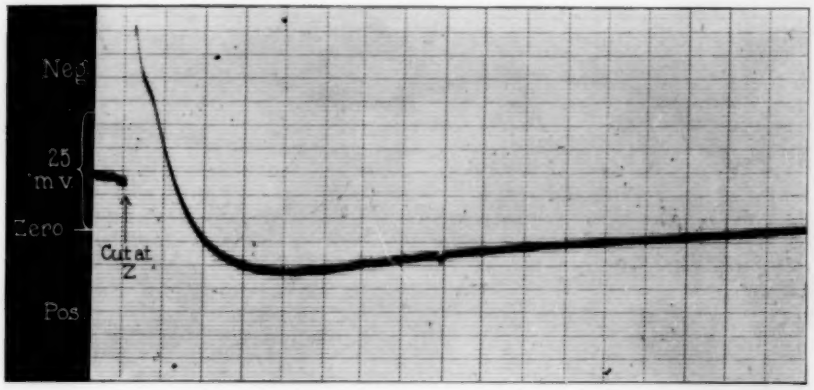

FIG. 12. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at *A* and *C*. At the start the curve shows a negative value of about 10 millivolts: ordinarily this would be near zero. On cutting at *Z*, *A* becomes more negative and then positive with respect to *C;* the potential difference then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

concentrated solution is the first to change. On this basis we may diagram the protoplasm as in Fig. 9, the arrows indicating the direction in which the positive current tends to flow and their length the relative magnitude (the direction of the *X* arrow is called negative by convention and that of the *Y* arrow positive; and since in this case the *X* arrow is longer the potential difference across the protoplasm is here said to be negative). If the value of the *X* arrow is x and that of the *Y* arrow is y we may assume that the observed potential difference is proportional to $y - x$.[1b] If both these values should fall off simul-

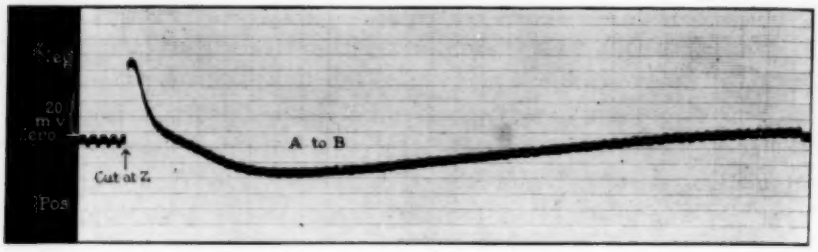

FIG. 13 *a*.

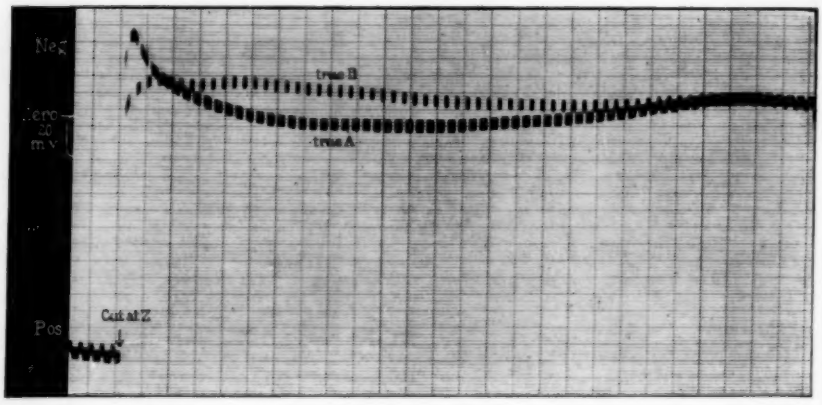

FIG. 13 *b*.

FIGS. 13 *a* and *b*. Photographic record of potential differences, the experiment being arranged as shown in Fig. 6 with 0.001 M KCl at *A*, *B*, and *C*. The record starts after *C* has been killed (by 0.001 M KCl saturated with chloroform) so that the curves for *A* and *B* have their "true" values (both are strongly positive and have about the same value so that they almost coincide; this high value is partly due to the fact that in this material 0.01 M is positive instead of being negative as usual: they become negative on cutting after which they approach zero); these are recorded on one instrument while the curve showing the potential difference between *A* and *B* (marked "*A* to *B*") is simultaneously recorded on another; the value of the latter is equal to the difference between the "true *A*" and "true *B*" (as illustrated in Figs. 14 *a* and *b*). The vertical marks represent 5 second intervals. Selected as typical from 25 experiments.

taneously, as shown in Fig. 10*b*, we should obtain the curve shown in Fig. 10*a* which is a tracing of the "true" *A* curve in Fig. 5.

Our hypothesis states that the layer in contact with the more concentrated solution will be the first to change and since 0.05 M KCl acts much like sap we might expect that when it is applied to the outside of the

FIG. 14 *a*.

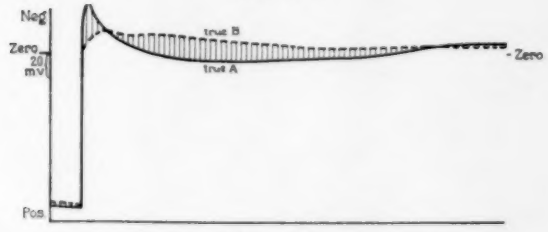

FIG. 14 *b*.

FIG. 14 *a*. Tracing of the curve in Fig. 13 *a* with vertical and horizontal scales made identical with those in Fig. 14 *b*. Certain ordinates are drawn for comparison with Fig. 14 *b*.

FIG. 14 *b*. Interpretation of Fig. 14 *a*. Fig. 14 *a* shows only the observed potential difference but Fig. 14 *b* shows that this is equal to the difference between the "true" curves of *A* and *B* (traced from Fig. 13 *b*); each ordinate is equal to the one directly above it in Fig. 14 *a*. At the start *A* is positive to *B* corresponding to the fact that the *A* to *B* curve of Fig. 14 *a* is positive (this curve shows the potential difference of *A* with reference to *B*): when *A* becomes negative to *B* the curve in Fig. 14 *a* becomes negative: *A* then becomes positive to *B* and then as *A* and *B* approach each other the curve in Fig. 14 *a* approaches zero.

cell *X* and *Y* would behave as if in contact with similar solutions and upon cutting would change at about the same rate, or that sometimes one and sometimes the other would go faster. This is actually the case: when there is a difference it is usually *Y* which goes first, just as in the case of chloroform,[16] so that the potential difference across the protoplasm first becomes more negative and then approaches zero (curve *B*, Fig. 11). Occasionally it would seem that *X* goes first,

giving curves like the *A* curve shown in Fig. 22*a* (see curve *A*, Fig. 11).[8]

Let us now consider the results obtained with a solution less concentrated than sap, *e.g.* 0.001 M KCl. We find (Fig. 12) that the cut makes *A* negative, then positive, after which the potential difference approaches zero (Fig. 12). When the experiment is arranged as in Fig. 6 we observe (Figs. 13*a* and *b*) after killing *C* that *A* and *B* are

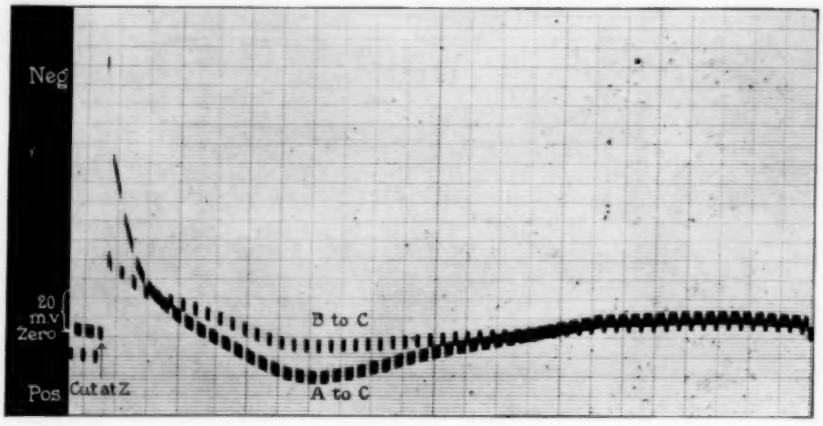

FIG. 15. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.001 M KCl at *A*, *B*, and *C*. On cutting at *Z* the *A* to *C* and *B* to *C* curves become negative, then positive, and the potential difference then approaches zero (the curves do not show the "true" values but only the potential difference with reference to *C*). The vertical lines represent 5 second intervals. *Cf.* Fig. 5. Selected as typical from 20 experiments.

positive (showing their "true" values).[1b] On cutting both become negative, after which they approach zero. It is evident that if we should lead off from *A* to *B* we should obtain approximately the difference between the "true" curves, giving a curve of the type seen in Fig. 12. This is the case, as seen in Fig. 13*a* and shown more clearly by Figs. 14*a* and *b*.

[8] The same cell may show both types of curves (as in Fig. 11) but in many cases but one is observed. The resulting *A* to *B* curves may resemble those shown in Figs. 2, 7 *b* or 22 *b*. The variations may be due to differences in the sap or in the protoplasm.

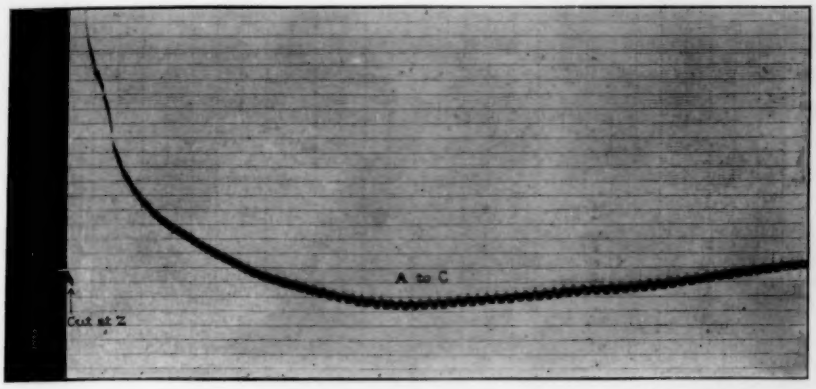

FIG. 16 *a*.

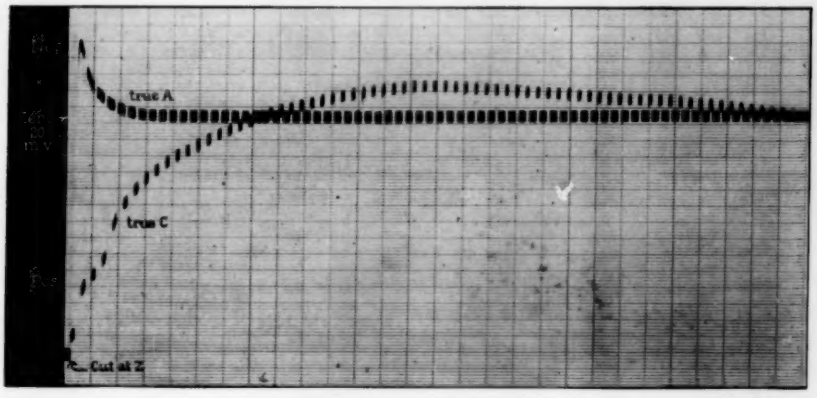

FIG. 16 *b*.

FIGS. 16 *a* and *b*. Photographic record of potential differences, the experiment being arranged as in Fig. 6 with 0.001 M KCl in contact with *A*, *B*, and *C*. The record starts after *B* has been killed (by 0.001 M KCl saturated with chloroform) so that the curves for *A* and *C* have their "true" values: before cutting both have about the same positive value (this is very high corresponding to the fact that with this material 0.01 M KCl is positive instead of being negative as usual) and coincide but after cutting they become negative and then approach zero; these are recorded on one instrument while the curve showing the potential difference between *A* and *C* (marked "*A* to *C*") is simultaneously recorded on another: the value of the *A* to *C* curve is equal to the difference between the "true *A*" and "true *C*" (*cf.* Figs. 14 *a* and *b*). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

If we should perform the experiment without killing *C* it would still be true (as previously explained) that the difference between the curves of *A* and *B* should give the curve observed in leading off from *A* to *B*. The result of such an experiment is shown in Fig. 15 and it is evident

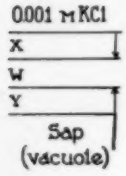

FIG. 17.

FIG. 17. Hypothetical diagram of the condition of the protoplasm when in contact with 0.001 M KCl. The value of the potential difference across the protoplasm is regarded by convention as positive since the *Y* arrow is longer.

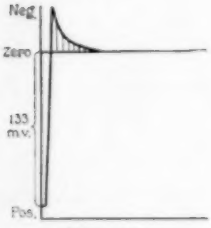

FIG. 18 *a*.

FIG. 18 *a*. Tracing of the "true *A*" curve shown in Fig. 16 *b*. Certain ordinates are drawn for comparison with Fig. 18 *b*.

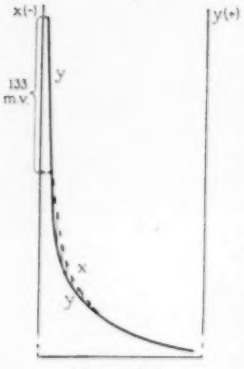

FIG. 18 *b*.

FIG. 18 *b*. Interpretation of Fig. 18 *a*. Fig. 18 *a* shows only the observed potential difference but Fig. 18 *b* shows that this is equal to the difference between the (hypothetical) value of *x* (broken line) which has a negative sign and the (hypothetical) value of *y* (unbroken line) with a positive sign: each ordinate is equal to the one directly above it in Fig. 18 *a*.

At the start the curve in Fig. 18 *a* is positive because the value of *y* (which is positive by convention) is greater. During the first period *y* falls until equal to *x* and the curve in Fig. 18 *a* is then at zero: when *y* becomes less than *x* the curve in Fig. 18 *a* becomes negative; the two curves then come together as the curve in Fig. 18 *a* approaches zero.

that if we pursue the scheme shown in Figs. 14*a* and *b* we shall have a similar result. In order to get the "true" *C* curve we employ the scheme shown in Fig. 6 and obtain the result in Figs. 16*a* and *b* (the *C* curves show considerable variation which will be discussed in a subsequent paper).

The next step is to interpret the "true" curves in 0.001 M KCl, such as the *A* curve in Fig. 13*b*. For this purpose we may diagram the protoplasm as in Fig. 17 making the *Y* arrow longer in order to show that the potential difference across the protoplasm is positive. If cutting

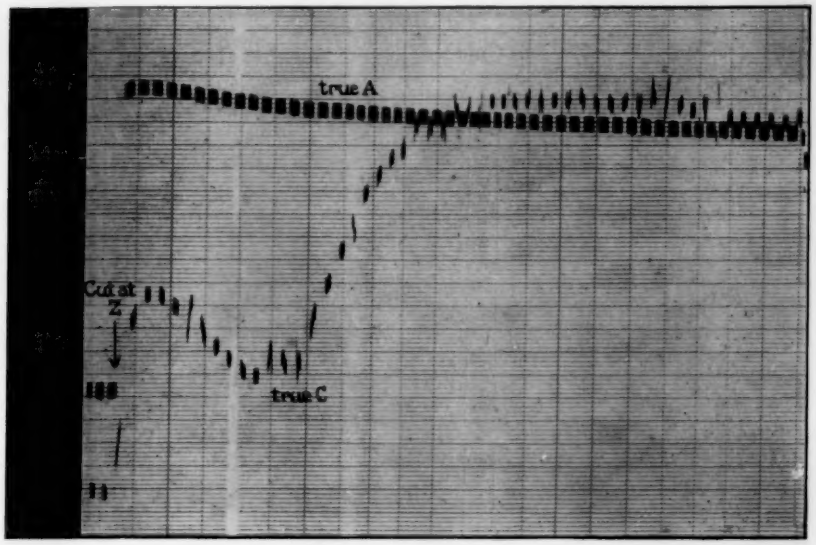

FIG. 19. Photographic record of potential differences, the experiment being arranged as shown in Fig. 6 with 0.001 M KCl at *A*, *B*, and *C*. When the record starts *B* has been killed (with 0.001 M KCl saturated with chloroform) so that the curves for *A* and *C* show their true values, *C* being more positive than *A* (the high positive values correspond to the fact that with this material 0.01 M KCl is positive instead of being negative as usual). On cutting at *Z* the curve of *A* becomes negative and then approaches zero: that of *C* becomes more negative, then more positive, then more negative, and then approaches zero (resembling curves obtained by killing with chloroform). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

caused the value of *y* to fall off more rapidly than that of *x* we might get the curve shown in Fig. 18*a* (which is a tracing of the "true" *A* curve of Fig. 16*b*). This would be expected on the basis of our hypothesis which states that in general the changes produced (by chloroform or by cutting) in any protoplasmic layer are more rapid the

higher the concentration of the salt solution in contact with it. As sap is approximately equivalent in these experiments to 0.05 M KCl it is

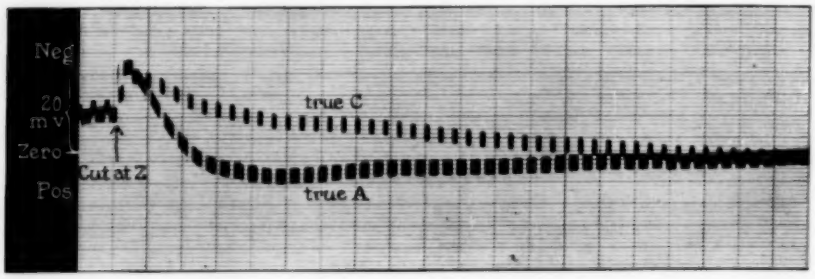

FIG. 20 *a*.

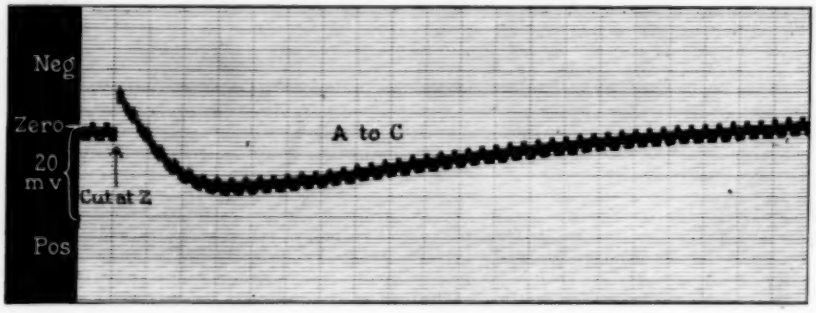

FIG. 20 *b*.

FIGS. 20 *a* and *b*. Photographic record of potential differences, the experiment being arranged as in Fig. 6 with 0.025 M KCl in contact with *A*, *B*, and *C*. The record starts after *B* has been killed (with 0.025 M KCl saturated with chloroform) so that the curves for *A* and *C* have their "true" values (both are negative at about the same value so that they almost coincide). On cutting at *Z* they become more negative and then approach zero (the process being more rapid at *A* than at *C*): these are recorded on one instrument while the curve showing the potential difference between *A* and *C* (marked "*A* to *C*") is simultaneously recorded on another; the value of the *A* to *C* curve is equal to the difference between the "true *A*" and "true *C*" (*cf.* Figs. 14 *a* and *b*). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

evident that in this case we should expect Y^{1b} which is in contact with sap to go before *X* which is in contact with 0.001 M KCl.

When we compare the "true" curves in Fig. 13*b* with those obtained (as described in a previous paper[1b]) by applying chloroform directly to *A* and *B* (instead of injuring them indirectly by cutting at *Z*) we see a general resemblance but they differ in details.[9] All that we can say at present is that the forms of these curves seem to depend on the relative rates of change of *x* and *y* without attempting to explain why cutting in many cases produces effects which are somewhat different from those observed with chloroform. (An example of another type

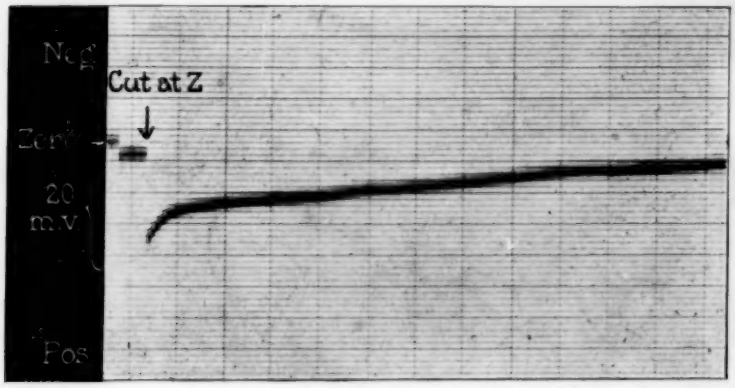

FIG. 21. Photographic record showing potential differences, the experiment being arranged as in Fig. 1 with 0.1 M KCl in contact with *A* and *C*. On cutting at *Z* the curve showing the potential difference of *A* with respect to *C* becomes more positive and the potential difference then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

The first mark is a zero mark.

of curve commonly found with chloroform is shown in the *C* curve in Fig. 19.[10])

Let us now consider concentrations (0.01 and 0.025 M KCl) which have a special interest because in some cases they produce a negative potential difference across the protoplasm. Our hypothesis predicts that in spite of this they will act on cutting like 0.001 M KCl which (prior to making the cut) shows a positive potential difference across the protoplasm. The following considerations show why this is so.

[9] Foot-note 1*b*, Fig. 12 *a*.

[10] Foot-note 1*b*, Fig. 9.

Since sap acts like 0.05 M KCl, 0.01 and 0.025 M KCl are less effective[11] solutions and we therefore expect the layer Y (which is in contact with sap) to go first; this would at first make the protoplasm more nega-

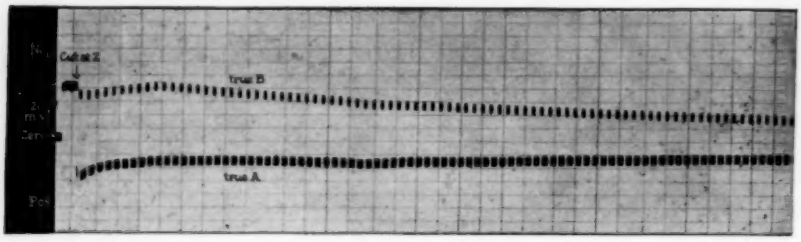

FIG. 22 *a*.

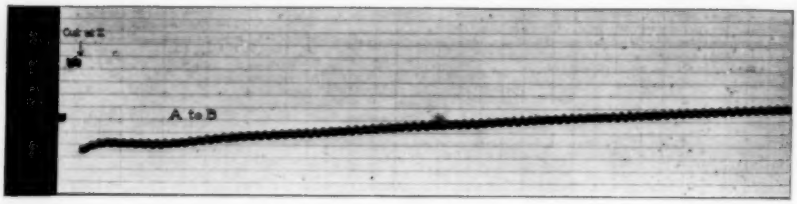

FIG. 22 *b*.

FIGS. 22 *a* and *b*. Photographic record showing potential differences, the experiment being arranged as in Fig. 6 with 0.1 M KCl at A, B, and C. The record starts (the first mark in Fig. 22 *a* is a zero mark and the first mark in Fig. 22 *b* is a calibration mark denoting 20 millivolts) after C has been killed (by 0.1 M KCl saturated with chloroform) so that the curves for A and B have their "true" values (both have the same negative value so that they coincide: on cutting they approach zero, A first becoming positive); these are recorded on one instrument while the curve showing the potential difference between A and B is simultaneously recorded on another (marked "A to B"); the value of the latter is equal to the difference between the "true A" and "true B." The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

tive (just as in the case of 0.001 M KCl). Fig. 20*a* shows that this is the case.

The interpretation of the "true" curves in Fig. 20*a* is like that of the curves obtained with 0.001 M KCl (*cf.* Figs. 18*a* and *b*).

[11] The word "effective" is used here in a technical sense. A solution is less effective than sap if it acts like dilute sap as tested by electrical criteria.

There is another case which should be considered, that of a solution more effective than sap (Fig. 21). Since in this case X is in contact with the more effective solution we should expect it to go first. This would make the protoplasm more positive after which the potential

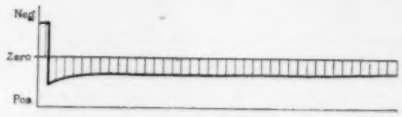

FIG. 23 *a*.

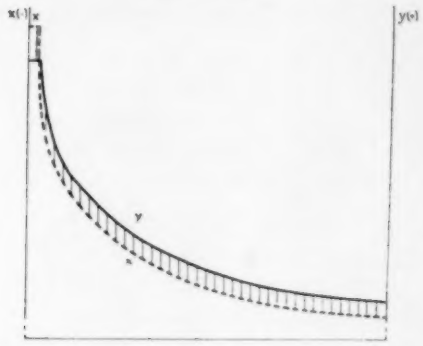

FIG. 23 *b*.

FIG. 23 *a*. Tracing of the "true" A curve shown in Fig. 22 *a* with certain ordinates drawn for comparison with Fig. 23 *b*.

FIG. 23 *b*. Interpretation of Fig. 23 *a*. Fig. 23 *a* shows only the observed potential difference but Fig. 23 *b* shows that this is equal to the difference between the (hypothetical) value of x (broken line) which has a negative sign and the (hypothetical) value of y (unbroken line) with a positive sign: each ordinate is equal to the one directly above it in Fig. 23 *a*.

At the start x is greater than y because the curve in Fig. 23 *a* is negative (and x is negative by convention): when x falls and becomes equal to y the curve in Fig. 23 *a* reaches zero; when x becomes less than y the curve in Fig. 23 *a* becomes positive.

difference would approach zero. That this is the case is shown in Fig. 22*a*. The behavior of the "true" B curve resembles that found with chloroform but in the case of the "true" A curve the first positive movement of the curve goes further and carries it past zero. The "true" curves show a positive drop at the start after which the potential difference approaches zero (in this case so slowly that only a part of

the curve is given): the process is more rapid and more pronounced at A than at B.

In this case we diagram the protoplasm as in Fig. 9 (but making the x arrow longer than in the figure) and interpret the "true" A curve in Fig. 22*a* in the manner shown in Figs. 23*a* and *b*.

These experiments confirm in a remarkable way the predictions of the hypothesis set forth in a previous paper[1b] and indicate that it may prove a useful guide.

We see that the injured protoplasm is sometimes positive and sometimes negative to uninjured protoplasm because the electromotive force of the dead protoplasm is nearly zero and its relation to normal protoplasm will depend on what solutions are employed. By making the concentration of KCl 0.01 or thereabouts we can always bring the living protoplasm of *Nitella* to the same potential difference as the dead protoplasm. If we use more concentrated KCl the injured spot will be positive to the uninjured one and with more dilute solutions it will be negative.[12]

Certain interesting phenomena connected with the death wave, *e.g.* the fact that it traverses the cell so rapidly and can pass over a killed spot (though unable to affect adjoining cells) as well as the fact that the death process is more rapid and more pronounced the greater its nearness to the cut, will be discussed in forthcoming papers.

SUMMARY.

Experiments on cutting confirm the prediction that the current of injury will be positive when the cell is in contact with concentrated solutions and negative with dilute solutions. They support the idea that the protoplasm is made up of layers differing considerably in their properties, each having a death curve of simple and regular form, the more rapid alteration of the outer layer making the protoplasm more positive and the more rapid alteration of the inner making it more negative.

From the point where the cell is cut a wave of some sort, which we may for convenience call a death wave, passes along the cell, setting up at each point it touches a death process which has the greater speed and intensity the nearer it is to the cut.

[12] *i.e.* always using identical solutions at both the injured and uninjured spots: otherwise there may be an effect due to the cell wall (concentration effect).

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